

UMAPORN PASTSART

ROLE OF ANTIOXIDANT ENZYMES IN MEAT OXIDATIVE STABILITY

Thesis submitted in fulfillment of the requirements
for the degree of Doctor (PhD) in Applied Biological Sciences

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Dutch translation of the title:

De rol van antioxidant enzymen in de oxidatieve stabiliteit van vlees.

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LIST OF ABBREVIATIONS

BF: *Musculus Biceps femoris*

CAT: catalase

DDC: diethyldithiocarbamate

DFD: dark, firm and dry meat

DNPH: 2,4-dinitrophenylhydrazine

G6PD: glucose 6-phosphate dehydrogenase

GR: glutathione reductase

GSH: glutathione

GST: glutathione S-transferase

GSH-Px: glutathione peroxidase

GS-SG: oxidized glutathione

IBF: inner *Musculus Biceps femoris*

ISM: inner *Musculus Semimembranosus*

LD: *Musculus Longissimus dorsi*

MDA: malondialdehyde

MetMb: metmyoglobin

MP: myofibrillar proteins

NADP: nicotinamide adenine dinucleotide phosphate

NADPH: reduced nicotinamide adenine dinucleotide phosphate

NaN₃: sodium azide

OBF: outer *Musculus Biceps femoris*

OSM: outer *Musculus Semimembranosus*

List of abbreviations

PCA: principal component analysis

pm: post-mortem

Pox: protein oxidation

PQM: pork quality meter

PSE: pale, soft and exudative meat

ROS: reactive oxygen species

SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis

SOD: superoxide dismutase

TBARS: thiobarbituric acid reactive substances

WHC: water-holding capacity

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GENERAL BACKGROUND

Meat has a special place in the diet of many people because of its appealing flavour and texture and its high nutritional value. However, there are numerous factors limiting the quality and acceptability of meat and meat products. Apart from microbiological hazards and possible contaminants, oxidation of lipid, myoglobin and protein is a major cause of quality deterioration of muscle foods. Lipid, pigment and protein oxidative processes in meat appear to be linked. The oxidation of one of these leads to the formation of chemical species that can exacerbate oxidation of the other (Chaijan, 2008; Faustman et al., 2010; Estévez, 2011). Oxidation of deoxymyoglobin to metmyoglobin causes brown discolouration in meat (Mancini & Hunt, 2005). Lipid oxidation leads to discolouration, drip losses, off-odour and off-flavour development, and decreases the nutritional quality and safety by the formation of secondary reaction products in foods after cooking and processing (Frankel, 1980; Morrissey et al., 1998). Protein oxidation leads to loss of activity of muscle proteases and the functionality of myofibrillar proteins (MP) (Xiong, 2000; Carlin et al., 2006) and reduced water-holding capacity (WHC), digestibility, and tenderness of meat (Bertram et al., 2007; Huff-Lonergan et al., 2010; Liu et al., 2010). Therefore, lowering these oxidations can enhance the shelf-life stability of meat and meat products.

Reactive oxygen species (ROS) such as the superoxide ($O_2^{\bullet-}$), the hydroperoxyl (HO_2^{\bullet}) and hydroxyl (HO^{\bullet}) radicals and other non-radical species such as the hydrogen peroxide

(H₂O₂) and hydroperoxides (ROOH) have been recognized as potential initiators of oxidation (Morrissey et al., 1998; Chaijan, 2008; Butterfield & Stadtman, 1997). There are several mechanisms to protect muscle in living animal to deal with those ROS and against oxidative processes, including the endogenous antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) (Chan & Decker 1994). The SOD is the first line antioxidant enzyme catalysing the dismutation of the highly reactive O₂^{•-} to O₂ and to the less reactive species H₂O₂. The resultant H₂O₂ is detoxified to O₂ and H₂O by CAT or GSH-Px (Matés et al., 1999). It is known that these enzymes display residual activity in muscle post-mortem (Renner et al., 1996; Pradhan, et al. 2000; Chen et al., 2010). However, it is unclear what is their contribution to the oxidative stability of meat during storage. Therefore, the main purpose of this doctoral research is to unravel the role of the three major antioxidant enzymes, namely SOD, GSH-Px, and CAT on lipid, myoglobin and protein oxidation in fresh meat of farm animals. In addition, we investigated the effect of muscle type and post-mortem rate of pH and temperature fall on the activity of these enzymes in relation to quality parameters such as colour, lipid oxidation and metmyoglobin formation in meat.

It is indeed known that variation in the rate of post-mortem glycolysis has an impact of the oxidative stability of meat (Juncher et al., 2001; Scheffler et al., 2011). In this respect, the influence of hot boning on antioxidant enzyme activities and oxidative stability of beef and pork muscles was investigated compared to conventional cooling and cold boning. Especially in beef inner and outer *Musculus Biceps femoris* muscles a fast pH fall and slow temperature decline in the deeper laying muscle induces heat shortening and protein denaturation, and results in a pale colour compared to the outer

layer and thus in an unattractive two-toning appearance of this muscle (Clinquart et al., 1998; De Smet, 2004; De Boever et al., 2009). Hot boning was therefore examined as a tool to overcome this problem (Sammel et al., 2002). The scientific interest of this thesis is in the investigation of the role of antioxidant enzymes in protection against oxidative processes in meat as affected by factors such as animal species, muscle type, and post-mortem glycolysis and temperature.

RESEARCH OBJECTIVES AND THESIS OUTLINE

The specific objectives of the present study are:

- 1) To investigate the potential role of antioxidant enzymes (superoxide dismutase, glutathione peroxidase, and catalase) on lipid, protein and myoglobin oxidation in meat;
- 2) To investigate the effect of muscle type, and the rate of post-mortem pH and temperature fall on the activity of these enzymes in relation to oxidation and meat quality parameters;
- 3) To study the effect of hot boning as a strategy for increasing the rate of chilling on antioxidant enzyme activities, oxidation and colour stability in meat.

This dissertation consists of six chapters (Fig. 1). **Chapter 1**, a literature overview on oxidative stress in animal tissues and antioxidant defense mechanisms is given. Additionally, a brief overview of meat quality and oxidation processes in meat is presented in this chapter. The experimental work is described in 4 chapters. **Chapter 2**

deals with the study of the potential role of antioxidant enzymes against oxidation in meat. **Chapter 3** describes the effect of muscle and post-mortem rate of pH and temperature fall on antioxidant enzyme activity in relation to other meat quality parameters in fresh beef. **Chapter 4** deals with the effect of high post-mortem temperature on antioxidant enzyme activities and meat quality in pork. **Chapter 5** discusses the effect of hot boning on colour stability, antioxidant enzyme activities and oxidation in beef inner and outer *M. Biceps femoris*. Finally, **Chapter 6** relates all data in a general discussion.

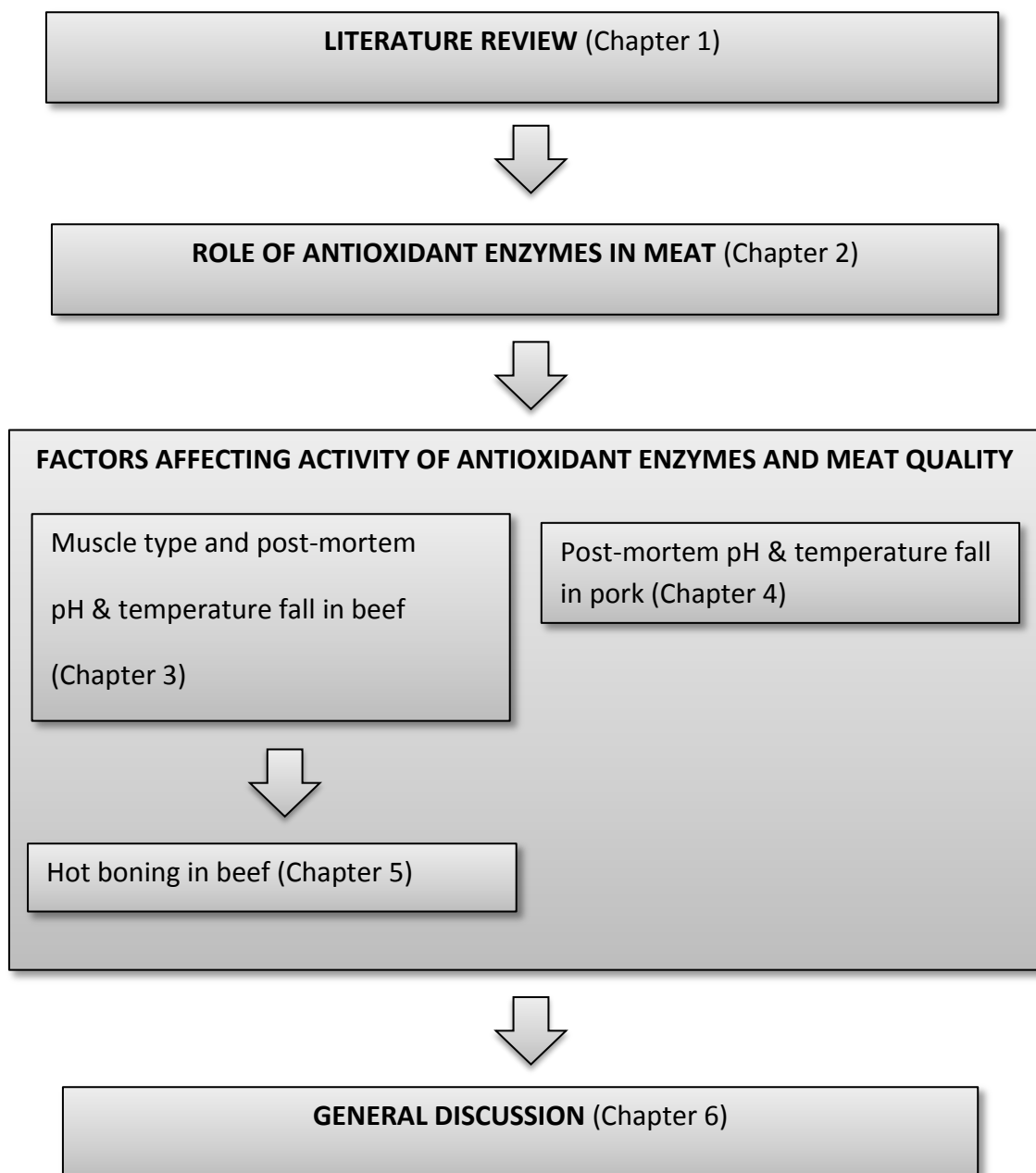


Fig. 1 Outline of the thesis

**LITERATURE BACKGROUND ON OXIDATIVE STRESS AND ANTIOXIDANT
DEFENSE IN THE LIVING ANIMAL AND OXIDATION IN MEAT**

LITERATURE BACKGROUND ON OXIDATIVE STRESS AND ANTIOXIDANT DEFENSE IN THE LIVING ANIMAL AND OXIDATION IN MEAT

1.1 OXIDATIVE STRESS AND ANTIOXIDANT DEFENSE IN THE LIVING ANIMAL

1.1.1 Oxidative stress

All aerobic organisms produce reactive oxygen species (ROS) as by-products of cell metabolism. Small amounts of ROS are continuously produced in cells for responding to both external and internal stimuli (Matés et al., 1999). Low concentrations of ROS are necessary in many biochemical processes (Birben et al., 2012). However, high amounts and/or insufficient removal of ROS results in **oxidative stress** (Matés et al., 1999), which may cause metabolic malfunctions and damage to biological macromolecules such as DNA, lipid, and protein. ROS can be divided into two groups: 1) free radical species such as the superoxide ($O_2^{\bullet-}$), the hydroperoxyl (HO_2^{\bullet}) and hydroxyl (HO^{\bullet}) radicals and 2) non radical species such as the hydrogen peroxide (H_2O_2) and hydroperoxides (ROOH). Some key points related to the sites of ROS production and antioxidant defense system in a typical mammalian cell are shown in Fig. 1.1.

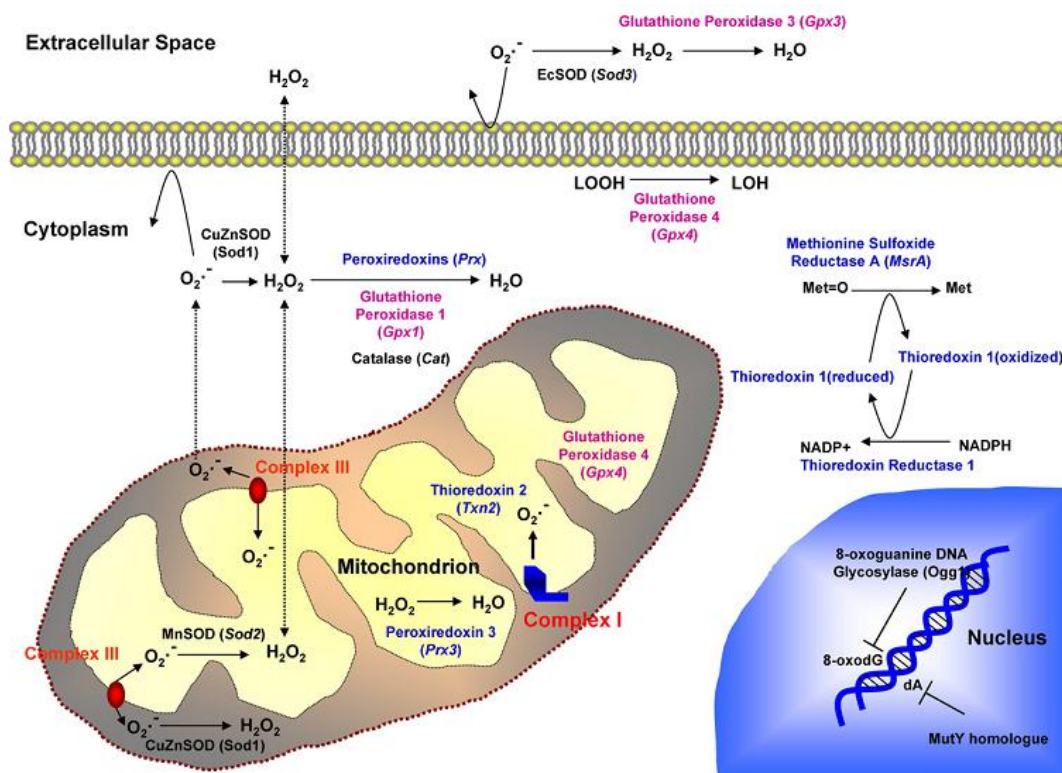


Fig. 1.1 Generation of reactive oxygen species (ROS) and the defense mechanisms against damage by antioxidant scavengers in mammalian cells (Muller et al., 2007)

Mitochondria are the major source of ROS (Fig. 1.1). Superoxide anion ($O_2^{\bullet-}$) is not membrane permeable while H_2O_2 is readily diffusible. Complex I releases $O_2^{\bullet-}$ exclusively into the mitochondrial matrix while Complex III can release $O_2^{\bullet-}$ to both sides of the inner mitochondrial membrane. If $O_2^{\bullet-}$ is released into the inter membrane space, it can reach the cytoplasm via the protein channels (Muller et al., 2007).

1.1.2 Antioxidant defenses

To minimize the damaging effects of ROS, aerobic organisms evolved both non-enzymatic and enzymatic antioxidant defenses.

1.1.2.1 Non-enzymatic antioxidants

Non-enzymatic antioxidants include low-molecular-weight compounds, such as vitamins (vitamins C and E), β -carotene, uric acid, and GSH, a tripeptide (L- γ - glutamyl-L-cysteinyl-L-glycine) that comprises a thiol (sulfhydryl) group (Birben et al., 2012).

Vitamin C (Ascorbic Acid)

Ascorbic acid is a small, water-soluble antioxidant molecule which acts as a primary substrate in the cyclic pathway for enzymatic detoxification of H_2O_2 . In addition, it acts directly to neutralize superoxide radicals, singlet oxygen or superoxide and as a secondary antioxidant during reductive recycling of the oxidized form of vitamin E (Noctor & Foyer, 1998).

Vitamin E (α -Tocopherol)

Lipid-soluble vitamin E is concentrated in the hydrophobic interior site of cell membranes and is the principal defense against oxidant-induced membrane injury. Vitamin E donates electrons to peroxyl radicals, which are produced during lipid peroxidation. The α -tocopherol is the most active form of vitamin E and the major membrane-bound antioxidant in cells (Birben et al., 2012).

Glutathione (GSH)

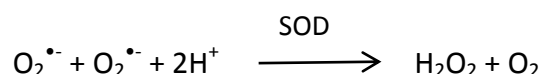
GSH is highly abundant in all cell compartments and is the major soluble antioxidant. The GSH/GS-SG (reduced to oxidized glutathione) ratio is a major determinant of oxidative stress. GSH shows its antioxidant effects in several ways. It detoxifies H_2O_2 and lipid peroxides via action of GSH-Px. GSH donates its electron to H_2O_2 to reduce it into H_2O and O_2 . GSH is a cofactor for several detoxifying enzymes, such as GSH-Px and transferase. It has a role in converting vitamin C and E back to their active forms. GSH protects cells against apoptosis by interacting with proapoptotic and antiapoptotic signaling pathways (Masella et al., 2005).

1.1.2.2 Enzymatic antioxidants

As can be seen in Fig. 1.1, the $\text{O}_2^{\bullet-}$ may be degraded or reduced into H_2O_2 in the mitochondrial matrix by Mn-SOD or, if it reaches the cytosol, by CuZn-SOD, while EC-SOD functions in the extracellular space. It is agreed upon that SODs comprise the main superoxide scavenging system in the cell, while catalase, glutathione peroxidase, and peroxiredoxins play an important role in the removal of H_2O_2 (Muller et al., 2007). Therefore, the major antioxidant enzymes that play an important role in protecting the cell from oxidative stress are 1) superoxide Dismutase (SOD), 2) Glutathione peroxidase (GSH-Px) and 3) catalase (CAT).

1.1.2.2.1 Superoxide Dismutase (SOD)

SOD (EC 1.15.1.1) is the most important and the first line of antioxidant enzyme defense system against ROS, particularly superoxide anion radical ($O_2^{\bullet-}$). SOD catalyses the dismutation of the highly reactive $O_2^{\bullet-}$ to O_2 and to the less reactive species H_2O_2 . Subsequently, peroxide can be reduced by catalase (CAT) or glutathione peroxidase (GSH-Px) (Matés et al., 1999).



There are three distinct isoforms of SOD in mammalian tissue, which are classified based on their catalytic center and/or their localization (Zelko et al., 2002).

1. Copper/zinc SOD (CuZn-SOD or SOD1) localized in the intracellular cytoplasmic compartments (McCords & Fridovich, 1969).
2. Manganese SOD (Mn-SOD or SOD2) localized in the mitochondria of aerobic cells (Weisiger & Fridovich, 1973).
3. Extracellular CuZn-SOD (EC-SOD or SOD3) localized in the extracellular elements (Marklund et al., 1982).

The genomic structure of three members of the human SOD enzyme family has been characterized. There is some amino acid sequence homology between CuZn-SOD and

EC-SOD, while Mn-SOD has no significant amino acid sequence homology with either CuZn-SOD or EC-SOD (Zelko et al., 2002).

Cytosolic CuZn superoxide dismutase (CuZn-SOD or SOD1) is a homodimeric metalloenzyme with a molecular weight of about 32 kDa. Each subunit contains a copper and a zinc atom at the active site bridged by a histamine residue. Copper atoms are involved in the catalytic activity, whereas zinc atoms are thought to play a role in maintaining the stability of CuZn-SOD (Fridovich, 1998). CuZn-SOD, which is the most abundant SOD enzyme in most tissues, is believed to play a major role in the first line of antioxidant defense (Marklund, 1980). CuZn-SOD is found predominantly in cytosol and in other compartments, such as nucleus and lysosomes of mammalian cells (Crapo et al., 1992; Liou et al., 1993).

Manganese superoxide dismutase (Mn-SOD or SOD2) is a homotetramer with a molecular weight of 96 kDa. Each subunit contains one manganese atom (Matés, 2000). Mn-SOD is found mainly in the mitochondrial matrix therefore Mn-SOD is present at the site of $O_2^{\bullet -}$ production (Weisiger & Fridovich, 1973). The contribution of Mn-SOD activity is dependent on the number of mitochondria. There is much evidence demonstrating that Mn-SOD is essential for the aerobic life, for example, Mn-SOD knockout mice die early after birth from cardiomyopathy and neurodegeneration (Lebovitz et al., 1996).

Extracellular CuZn superoxide dismutase (EC-SOD or SOD3) is the most recently discovered and least characterized member of the SOD family. EC-SOD is a hydrophobic glycoprotein with a molecular weight of approximately 135 kDa. In most species, the enzyme exists as a homotetramer of identical 30 kDa subunits, which contains one Cu and one Zn atom per subunit and both are required for enzymatic activity (Marklund,

1982). This enzyme is found predominantly in the extracellular matrix, e.g. plasma, serum, lymph, ascites and cerebrospinal fluid of mammalian tissues (Marklund, 1984a).

SOD activity can be inhibited by diethyldithiocarbamate (DDC), an effective chelating agent for Cu. It was found that DDC reversibly inactivates SOD *in vivo* (Heikkila et al., 1976) and *in vitro* (Heikkila et al., 1976, Misra, 1979). Sohal et al. (1984) reported that DDC can inhibit total SOD activity *in vivo*, but Iqbal and Whitney (1991) subsequently indicated that DDC treatment did not cause a significant loss of Mn-SOD activity *in vitro*. The inhibition reaction between DDC and SOD occurs in two distinct phases. First, a DDC molecule reacts with a Cu center with retention of enzyme activity in Phase I, and then a second DDC molecule displaces the Cu with a loss of activity in Phase II (Misra, 1979).

1.1.2.2.2 Glutathione peroxidase (GSH-Px)

Glutathione peroxidases (GSH-Pxs) (EC 1.11.1.19) are a family of enzymes that can be divided into two groups, 1) selenium-independent and 2) selenium-dependent enzymes. At present, 8 GSH-Pxs have been identified so far in mammals. GSH-Px1, 2, 3 and 4 are selenoproteins with a selenocysteine (Sec) in the catalytic center. They are the first GSH-Px enzymes that have been identified in mammalian cells. GSH-Px6 is also a selenoprotein but is present only in humans and pigs. GSH-Px5, 7 and 8 do not contain selenium at the active site (Brigelius-Flohé & Maiorino, 2013). GSH-Px protects mammalian cells against oxidative damage by catalyzing the reduction of H₂O₂ or organic hydroperoxides to H₂O or the corresponding alcohols, respectively at the expense of oxidizing GSH to its disulfide form (GS-SG).

Reduced glutathione (GSH) is used as a co-substrate to metabolize H_2O_2 or hydroperoxides (ROOH), resulting in H_2O and GS-SG (oxidized glutathione). GS-SG can be reduced back to GSH by the enzyme GSH reductase (GR), a reaction requiring NADPH regenerated by glucose 6-phosphate dehydrogenase (G6PD). The capacity to recycle GSH makes the GSH cycle a pivotal antioxidant defense mechanism for cells and prevents the depletion of cellular thiols (Fig. 1.2).

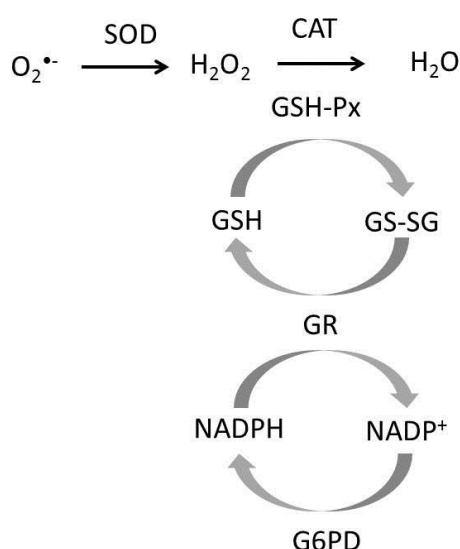


Fig. 1.2 Glutathione redox cycle. GSH-Px = Glutathione peroxidase, GSH = Glutathione, GS-SG = Oxidized glutathione, GR = Glutathione reductase, NADP = Nicotinamide adenine dinucleotide phosphate, NADPH = Reduced nicotinamide adenine dinucleotide phosphate, G6PD = Glucose 6-phosphate dehydrogenase. Modified from Heffner and Repine (1989)

There are four well known GSH-Pxs containing selenium at the active site in mammalian tissues:

- 1) The classical glutathione peroxidase (cGSH-Px or GSH-Px1)
- 2) The gastrointestinal glutathione peroxidase (GI-GSH-Px or GSH-Px2)
- 3) The plasma glutathione peroxidase (pGSH-Px or GSH-Px3)
- 4) The phospholipid hydroperoxide glutathione peroxidase (PHGSH-Px or GSH-Px4)

The classical or cytosolic glutathione peroxidase (GSH-Px1) was first described in 1957, and its function was hypothesized to be protection of red blood cells against hemolysis by oxidation (Mills, 1957). The GSH-Px1 is a homotetramer and reacts with H_2O_2 and soluble low molecular mass hydroperoxides, but not with hydroperoxides of more complex lipids, which is the function of GSH-Px4 (Marinho et al., 1997). Due to its hydroperoxide-reducing capacity, GSH-Px1 was classified as the enzyme counteracting oxidative stress. The GSH-Px1 is found ubiquitously intracellular and localized mainly in the cytosol, but some GSH-Px1 activity has been found also in the mitochondria (Mbemba et al., 1985; Esworthy et al., 1997).

The gastrointestinal glutathione peroxidase (GI-GSH-Px or GSH-Px2) is a second form of GSH-Px and found in the cytosol. This tetrameric enzyme has approximately 65% amino acid sequence identity and 60% nucleotide sequence identity with GSH-Px1. Both GSH-Px1 and GSH-Px2 have similar substrate specificity. They reduce H_2O_2 or fatty acid hydroperoxides rapidly, but do not reduce phospholipid hydroperoxides. Because GSH-Px2 is mainly expressed in the gastrointestinal system including the epithelium of the esophagus and, in humans, also in the liver, it was named GI-GSH-Px. It functions as a barrier against absorption of food-born hydroperoxides (Chu et al., 1993).

The plasma or extracellular glutathione peroxidase (pGSH-Px or GSH-Px3) is a major enzyme in reducing lipid hydroperoxides and H_2O_2 in plasma. GSH-Px3 is also similar to GSH-Px1 which is a tetramer. It was first detected in blood plasma and milk and is secreted *in vitro* by a number of cells and cell lines. It is a glycoprotein, which is consistent with an extracellular function (Avissar et al., 1994). Although GSH-Px3 is synthesized in a range of tissues, the major source for plasma is in the kidney. The importance of kidney as a source of GSH-Px3 has been emphasized by the localization of its mRNA in the epithelial cells of the proximal tubules in humans, rats and mice (Arthur, 2000).

Phospholipid hydroperoxide glutathione peroxidase (PHGSH-Px or GSH-Px4) is the fourth selenium-containing GSH-Px that has been characterized. GSH-Px4 was first purified in 1982 by Ursini and co-workers and described as a peroxidation-inhibiting protein (Ursini et al., 1995). There are many differences between GSH-Px4 and others (GSH-Px1, 2 and 3). The major structural difference is that GSH-Px4 is a monomer in contrast to the tetrameric structure of the others. GSH-Px4 consists of a mitochondrial form of approximately 22 kDa and a non-mitochondrial form of approximately 19 kDa. Moreover, GSH-Px4 can use phospholipid hydroperoxides as substrate as well as hydrogen peroxide and a wide range of other lipid hydroperoxides. Phospholipid hydroperoxides have the potential to cause damage in the cell membrane. GSH-Px1 cannot react directly with phospholipid hydroperoxides unless they are first metabolized by phospholipase A_2 . Hence, it is clear that GSH-Px4 is responsible for protection against oxidative damage of membranes. However, it is also certain that this enzyme has many more functions in controlling the metabolism of lipid peroxides (Arthur, 2000).

Additionally, nowadays, there are at least four other proteins, namely GSH-Px5, 6, 7 and 8, which have been recently identified in mammalian tissues (Brigelius-Flohé & Maiorino, 2013). GSH-Px5 is an epididymis-specific CysGSH-Px in mice, rats, pigs, monkey and human and similar in nucleotide/protein sequence with GSH-Px3, but it lacks selenocysteine at the active site (Ghyselinck & Dufaure, 1990). GSH-Px6 has been identified recently as a selenium-containing GSH-Px in human and pig, having structural similarity with GSH-Px4, but has been found to be inactive in mouse (Kryukov et al., 2003). Since, to date, GSH-Px family enzymes have been studied mainly in human and mouse/rat, it is appropriate to investigate their structures and tissue distributions in other animals (Fukuhara & Kageyama, 2005). The overall characterizations of 8 GSH-Pxs enzymes have been shown in the Table 1.1.

GSH-Px can be inactivated by misonidazole (Kumar & Weiss, 1986), mercaptosuccinate, penicillamine and α -mercaptopropionylglycine. The latter three compounds are currently used as slow-acting drugs in the treatment of rheumatoid arthritis. Chaudiere et al. (1984) indicated that mercaptosuccinate appears to be the most effective inhibitor and may not affect other biological components. Their results supported the formation of reversible enzyme-inhibitor complexes. The active site selenocysteine was trapped by the rapid binding of the inhibitor in competition with GSH which was consistent with the formation of thioselenate adducts of the active site. The model that best described the inhibition implies that a selenenic acid is not formed when hydroperoxide is reduced.

Table 1.1 Structural features and proven substrates of vertebrate GSH-Pxs (Brigelius-Flohé & Maiorino, 2013)

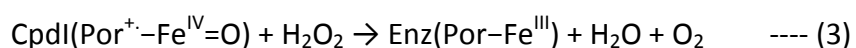
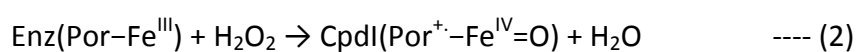
Mammalian GSH-Px type	Peroxidatic residue	Homo-tetramer	Reducing substrate	Oxidizing substrates
GSH-Px1	Sec	Yes	GSH	H ₂ O ₂ , soluble LOOH ROOH Peroxynitrite
GSH-Px2	Sec	Yes	n.d.	n.d.
GSH-Px3	Sec	Yes	GSH Low rate with Trx and Grx	H ₂ O ₂ , soluble LOOH, ROOH, PLOOH
GSH-Px4	Sec	No	GSH, DTT Mercaptoethanol, Cysteine particular protein thiols	H ₂ O ₂ , LOOH ROOH, PLOOH, ChOOH, CEOOH Peroxynitrite
GSH-Px5	Cys	Yes	n.d.	n.d.
GSH-Px6	Sec in humans, Cys in rats and mice	Yes	n.d.	n.d.
GSH-Px7	Cys	No	GSH, PDI	H ₂ O ₂
GSH-Px8	Cys	No	GSH, PDI?	H ₂ O ₂

ChOOH, cholesterol hydroperoxide; CEOOH cholesterol ester hydroperoxide; DTT, dithiotreitol; Grx, glutaredoxin; LOOH, lipid (fatty acid) hydroperoxide; PLOOH, phospholipid hydroperoxide of different classes; ROOH, small synthetic hydroperoxides (e.g. cumene hydroperoxide and tert-butyl-hydroperoxide); Trx, thioredoxin; PDI, protein disulfide isomerase; n.d., not determined

1.1.2.2.3 Catalase (CAT)

Catalase (EC 1.11.1.6) is a cytoplasmic enzyme that catalyzes the reduction of H₂O₂ to H₂O and O₂ (as Reaction 1) thereby preventing the accumulation of H₂O₂ and the toxic consequences of the reaction of H₂O₂ with other cellular components. The catalytic

reaction takes place in two steps with the first H_2O_2 molecule oxidizing the heme to an oxyferryl species in which one oxidation equivalent is removed from the iron and one from the porphyrin ring to generate a porphyrin cation radical (Reaction 2). The second H_2O_2 is then used as a reductant of compound I to regenerate the resting state enzyme, H_2O and O_2 (Reaction 3) (Switala & Loewen, 2002).



Catalases have been isolated from a broad range of prokaryotic and eukaryotic organisms. An evolutionary based classification of catalases has been proposed, dividing these enzymes into three subgroups possessing distinct structural and functional properties, namely 1) typical catalases, 2) catalases-peroxidases, and 3) manganese catalases (Zámocký & Koller, 1999).

The first and second subgroups are heme-containing enzymes, whereas the third subgroup contains manganese in their active sites. Most of the eukaryotic catalases described belong to the typical catalases subgroup. Typical catalase is the largest subgroup and found in almost all aerobically respiring organisms, both prokaryotes and eukaryotes. Most of these hydroperoxidases are homotetramers, 200–340 kDa in size with four prosthetic haem groups. The prosthetic haem group is localized at the center of the globular structure. The subunit forms a characteristic globule with an extended N-terminal arm (Zámocký & Koller, 1999). Catalase-peroxidases can be found in all three

living kingdoms, although in eukaryotes they were detected only in fungi (Fraaije et al., 1996). Manganese catalases are sometimes referred to as pseudocatalases. There are 3 manganese catalases known, one from lactic acid bacteria and two from thermophilic organisms (Zámocký & Koller, 1999).

The activity of catalase can be inhibited by several compounds, such as hydroxylamine (Kulys et al., 2003), 3-Amino-1:2:4-Triazole (Margoliash & Novogrodsky, 1958), cyanide, azide (Blaschko, 1935) and its natural substrate, H_2O_2 (concentrations $> 0.1 \text{ M}$ and reaction times $> 30 \text{ s}$) (Aksoy et al., 2004). It was found that sodium azide (NaN_3) is a very powerful inhibitor of CAT, and its action is completely reversible (Blaschko, 1935). Aksoy et al. (2004) proposed the reaction of catalase with H_2O_2 and azide as shown in Fig. 1.3. The enzyme can function in 2 ways: catalytically, decomposing H_2O_2 into H_2O and O_2 (α -phase), or peroxidatively, by eliminating H_2O_2 through oxidation of alcohols, formate or nitrate (β -phase).

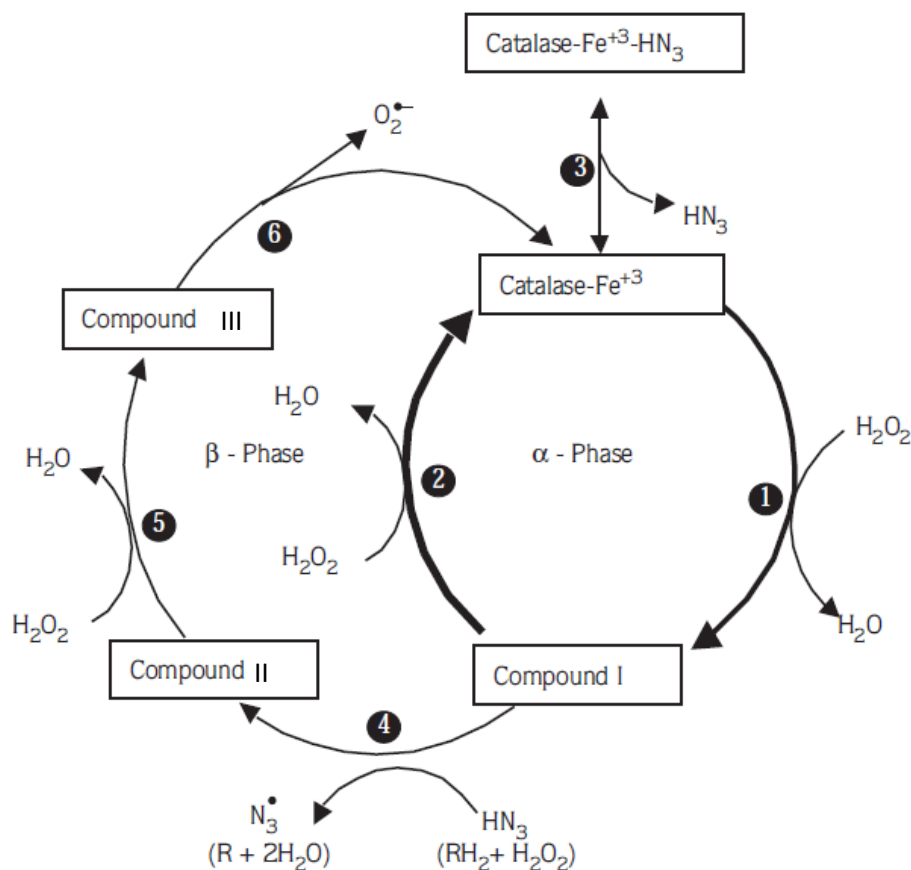


Fig. 1.3 Reaction of human red blood cell catalase with hydrogen peroxide and azide (e.g., RH₂: Formic acid, Methanol, Ethanol) (Aksoy et al., 2004)

1.2 MEAT QUALITY AND OXIDATION IN MEAT

1.2.1 Meat quality

Warriss (2010) defined that meat quality can be distinguished into two types; functional quality and conformance quality. Functional quality refers to desirable attributes in a product. Conformance quality is producing a product that meets the specification of the consumer exactly. A list of meat quality characteristics are shown in Table 1.2.

Table 1.2 The major components of meat quality (Warriss, 2010)

Yield and gross composition	Quantity of saleable product Ratio of fat to lean Muscle size and shape
Appearance and technological characteristics	Fat texture and colour Amount of marbling in lean (intramuscular fat) Colour and water - holding capacity of lean Chemical composition of lean
Palatability	Texture and tenderness Juiciness Flavour
Wholesomeness	Nutritional quality Chemical safety Microbiological safety
Ethical quality	Acceptable husbandry of animals

1.2.1.1 Factors affecting meat quality

1.2.1.1.1 Colour

Meat colour is the first criterion to judge meat quality and acceptability by consumers (Mancini & Hunt, 2005). Meat colour is mainly influenced by two main factors. The first is the concentration and state of myoglobin (Mb); the second is the meat structure (Warriss, 2010). The latter is directly linked to the ultimate pH (Abril et al., 2001).

Myoglobin is the water-soluble protein responsible for meat colour containing 8 α -helices linked by short non-helical sections. It contains a prosthetic group located within the protein's hydrophobic heme pocket. The heme ring has an iron atom at the center which can form six bonds. Four of these bonds link with pyrrole nitrogens, while the 5th arm coordinates with the proximal histidine-93 and the 6th arm is available to reversibly bind ligands including diatomic oxygen, carbon monoxide, water, and nitric oxide (Fig.

1.4). The concentration of myoglobin can be affected by both genetics and environment (Renner, 1990).

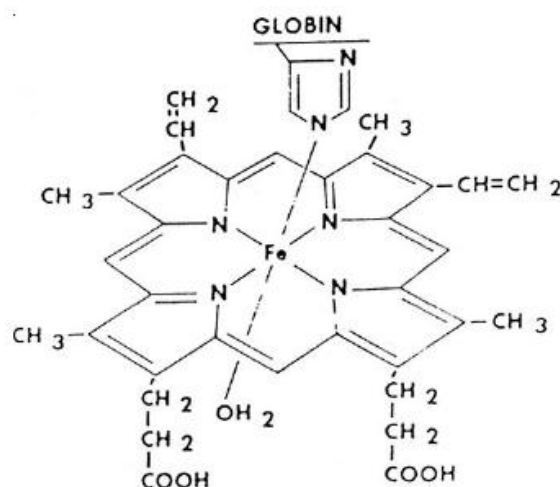


Fig. 1.4 Chemical structure of myoglobin (Pearson & Young, 1989)

The ligand present at the 6th coordination site and the valence state of iron determine meat colour via four chemical forms of myoglobin, deoxymyoglobin, oxymyoglobin, carboxymyoglobin and metmyoglobin (Fig. 1.5).

Deoxymyoglobin occurs when no ligand is present at the 6th coordination site and the heme iron is ferrous (Fe^{2+}) and gives meat the purplish-red or purplish-pink colour. Very low oxygen tension (<1.4 mm Hg) is required to maintain myoglobin in a deoxygenated state, such as in vacuum packaged meat or meat just after cutting (Mancini & Hunt, 2005). **Oxymyoglobin** occurs when myoglobin is exposed to oxygen (oxygenation) and development of a bright cherry red colour which the consumers associate with fresh meat. Oxymyoglobin has diatomic oxygen attached to the 6th coordination site of ferrous iron (Fe^{2+}). The oxygen ligand also interacts with the distal histidine-64, producing a

more compact protein structure than deoxymyoglobin, which has no ligand present to link iron to the distal histidine. Oxymyoglobin penetrates deeper into the surface of meat with increased exposure to oxygen. Depth of oxygen penetration and thickness of the oxymyoglobin layer depend on the meat's temperature, oxygen partial pressure, pH, and competition for oxygen by other respiratory processes. **Metmyoglobin** is the oxidized tan to brown coloured form of myoglobin and it contains ferric iron (Fe^{3+}). Normally, metmyoglobin forms easily at low oxygen partial pressure, or there is oxygen consumption (<7 mm Hg or about 1 to 2% oxygen) and water is the ligand at the 6th position of the iron in metmyoglobin (AMSA, 2012). Discolouration results from oxidation of both ferrous myoglobin derivatives to ferric iron ($\text{Fe}^{2+} \rightarrow \text{Fe}^{3+}$). Metmyoglobin beneath the surface of the meat, located between superficial oxymyoglobin and interior deoxymyoglobin, can gradually thicken and move towards the surface (Mancini & Hunt, 2005). **Carboxymyoglobin** formation occurs when carbon monoxide attaches to the vacant 6th position of deoxymyoglobin, producing a stable bright-red colour when the environment is devoid of oxygen. Atmospheres containing oxygen (albeit concentration dependent) will result in the conversion of carboxymyoglobin to either oxymyoglobin or metmyoglobin (AMSA, 2012).

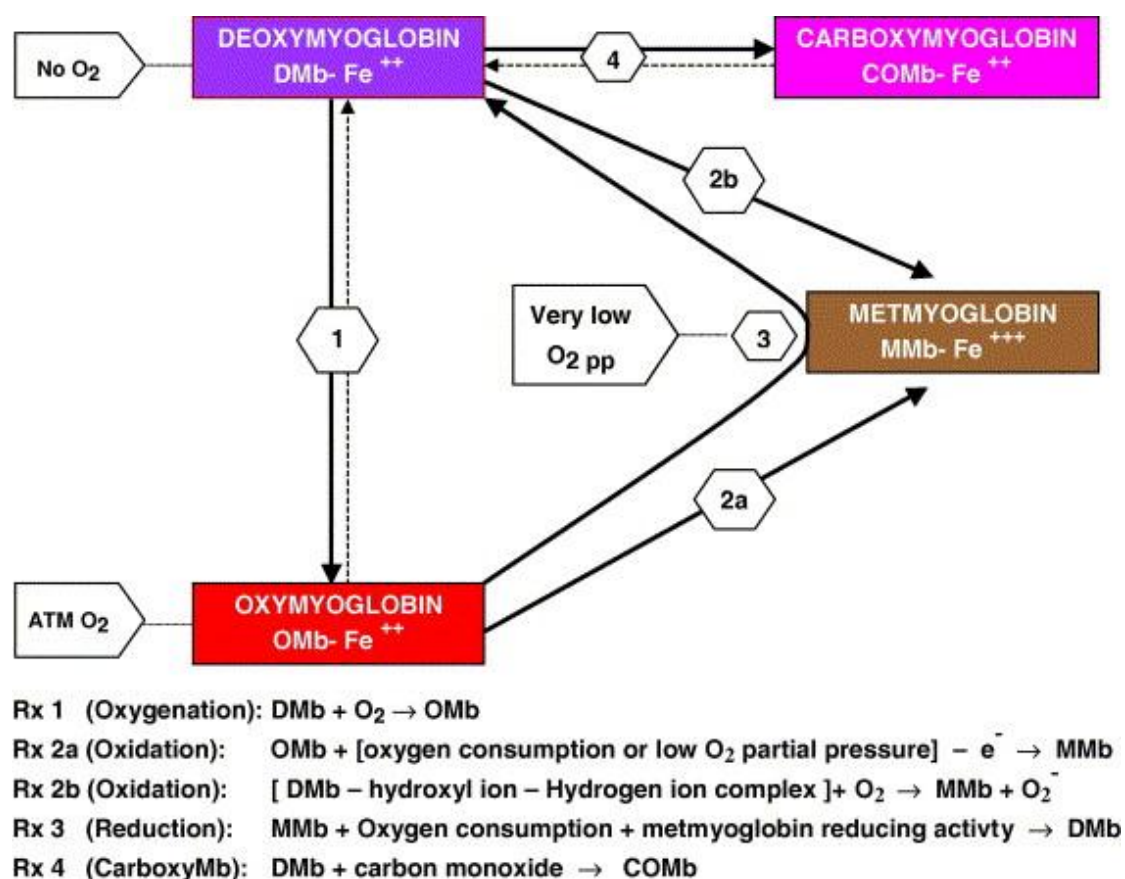


Fig. 1.5 Visible myoglobin redox interconversions on the surface of meat (Mancini & Hunt, 2005)

Another factor which has a great impact on the colour of meat is the rate and extent that muscle pH and temperature declines post-mortem. An increasing paleness in meat is inversely proportional to pH. If the ultimate pH of meat is high, e.g. when glycogen depletion occurs pre-slaughter resulting in little or no lactic acid production, the meat will be darker (Bendall & Swatland, 1988).

The colour of meat can be evaluated by visual appraisal or instrumentally by reflectance measurements (AMSA, 2012). Colour coordinates are generally expressed as C.I.E. L^*

(lightness), a^* (redness) and b^* (yellowness) values. Derived indices are hue angle and saturation index (or chroma).

1.2.1.1.2 Water holding capacity

Water holding capacity (WHC) is the ability of meat to bind its own or added water during the application of any force (Hamm, 1986). When meat loses water it is known as **drip loss**. Lean muscle contains approximately 75% water. The other main components include protein (approximately 20%), lipids or fat (approximately 5%), carbohydrates (approximately 1%) and vitamins and minerals (approximately 1%). The majority of water in muscle is held within the structure of the muscle and muscle cells. Specifically, within the muscle cell, water is found within the myofibrils, between the myofibrils themselves and between the myofibrils and the cell membrane (sarcolemma), between muscle cells and between muscle bundles (groups of muscle cells) (Offer & Cousins, 1992). In fact, there are three kinds of water found in muscle. The first is bound water which is found close to non-aqueous constituents like proteins and does not easily move to other compartments. This water is very resistant to freezing and to be driven off by conventional heating. The second is entrapped or immobilized water which is held either by steric effects or by attraction to the bound water. This water is held within the structure of the muscle but is not bound to the protein. This water does not flow freely from the tissue early post-mortem but it can be removed by drying, and can be easily converted to ice during freezing. The third is free water whose flow from the tissue is unimpeded. This fraction of water is held to the meat by weak surface forces (Huff-Lonergan & Lonergan, 2005). Immobilized water is most affected by the rigor process

and the conversion of muscle to meat. Due to alteration of muscle cell structure and lowering of the pH, this water can also be affected (Offer & Knight, 1988). The pH has a large effect on WHC. During the conversion of muscle to meat, WHC will be decreased. The rate at which pH falls as well as the ultimate pH of the meat will have an effect on this. The higher the ultimate pH, the higher the water holding capacity will be. A fast rate in pH decline, as well as a fast rate of pH decline at high temperature, will both result in a loss of WHC. This can be attributed to the denaturation of muscle proteins, in particular myosin (Offer, 1991).

1.2.1.1.3 The muscle pH and temperature relationship

The pH temperature relationship is one of the most important factors that can influence the process of the conversion of muscle to meat. Muscle pH and temperature interact continuously during rigor development, as they impact on both physical shortening (Tornberg, 1996), and proteolytic enzyme activity (Koohmaraie, 1992). The rate of pH drop post-mortem is inversely related to meat tenderness, with a slower fall in pH resulting in more tender meat (Hwang & Thompson, 2001a). Locker and Hagyard (1963) described that muscle shortening occurs when pre-rigor muscle is held at either low or high temperatures. Cold shortening is the result of the rapid chilling of carcasses immediately after slaughter, before the onset of rigor and the subsequent meat is tough after cooking. The muscle pH has to be greater than 6 at a temperature below 10 °C and still have ATP (Adenosine triphosphate) available for muscle contraction. The mechanism of cold shortening can be stimulated by low temperature and the massive release of Ca ions from the sarcoplasmic reticulum (SR) without subsequent

sequestration because the Ca pump of the SR does not function very well at low temperature. The Ca ions can activate the actomyosin ATP-ase and result in muscle contraction (Warriss, 2010). The contraction of muscle that is not followed by relaxation leads to short sarcomeres and tougher meat (Marsh & Leet, 1966). Heat shortening is caused by a combination of a high temperature with a low pH. The muscles are stimulated and allowed to contract and shorten at high temperature without subsequent relaxation and the subsequent meat is tough, particularly in beef (Locker & Daines, 1975). Both cold and heat shortening lead to decreased tenderness and increased drip loss (Thompson, 2002). A good relationship between pH and temperature seems to be a pH of more than 6 at temperatures above 35 °C and a pH below 6 for temperatures below 12 °C (Thompson, 2002). Electrical stimulation can have an effect on the pH/temperature relationship. Electrical stimulation can prevent cold shortening by causing a faster drop in pH in cases where carcasses are chilled rapidly or hot-deboning occurs (Davey et al., 1976; Hwang & Thompson, 2001b). However, over electrical stimulation can cause heat shortening in meat resulting from pH fall too fast but muscle temperature is still high (Warriss, 2010).

1.2.1.2 Major quality problems in beef and pork

1.2.1.2.1 Pale, soft and exudative (PSE) and dark, firm and dry (DFD) meat

PSE and DFD meats are two of the major quality problems in meat industry (Cassens, 2000). At present, it can be suggested that PSE and DFD conditions can occur in all species depending on how animals are handled ante-mortem (Adzitey & Nurul, 2011).

The appearances of typical PSE and DFD compared to normal meat are illustrated in Fig. 1.6.

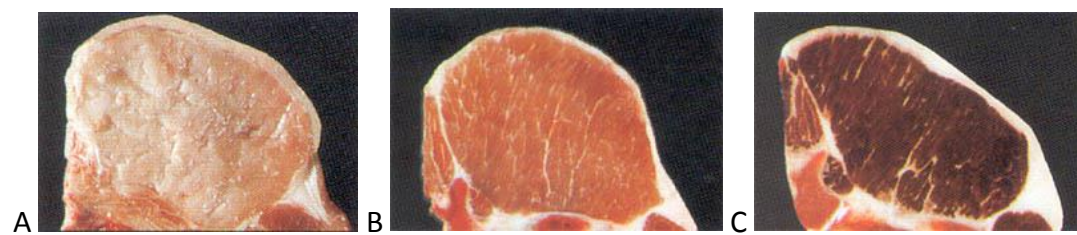


Fig. 1.6 A) PSE meat B) normal meat and C) DFD pork (FAO, 2014)

PSE and DFD meats are defined by the pH value of the meat at specific times. PSE is normally defined as having a pH at 45 min after slaughter lower than < 6 . DFD (also known as dark cutting in beef) is when the ultimate pH post-mortem measured after 12 – 48 hours is ≥ 6 (Table 1.3).

Table 1.3 Typical pH value limits for PSE, normal and DFD pork *M. Longissimus dorsi* (Warriss, 2010)

	PSE	Normal	DFD
pH ₄₅	<6.0	6.4	6.4
pH _u	5.3	5.5	≥ 6.0

The most common factor leading to both PSE and DFD in meat is stress ante-mortem. Exposure of animals to acute stress, e.g. the use of electric goads, fighting among animals, beating of animals prior to slaughter and overcrowding in the lairage, may result in PSE meat (Adzitey & Nurul, 2011). In addition, PSE mainly affects pig carcasses

and can be influenced by genetic factors, zootechnical management and environment conditions (Martinez-Rodriguez et al., 2011). The factors that can cause PSE meat are shown in Fig. 1.7.

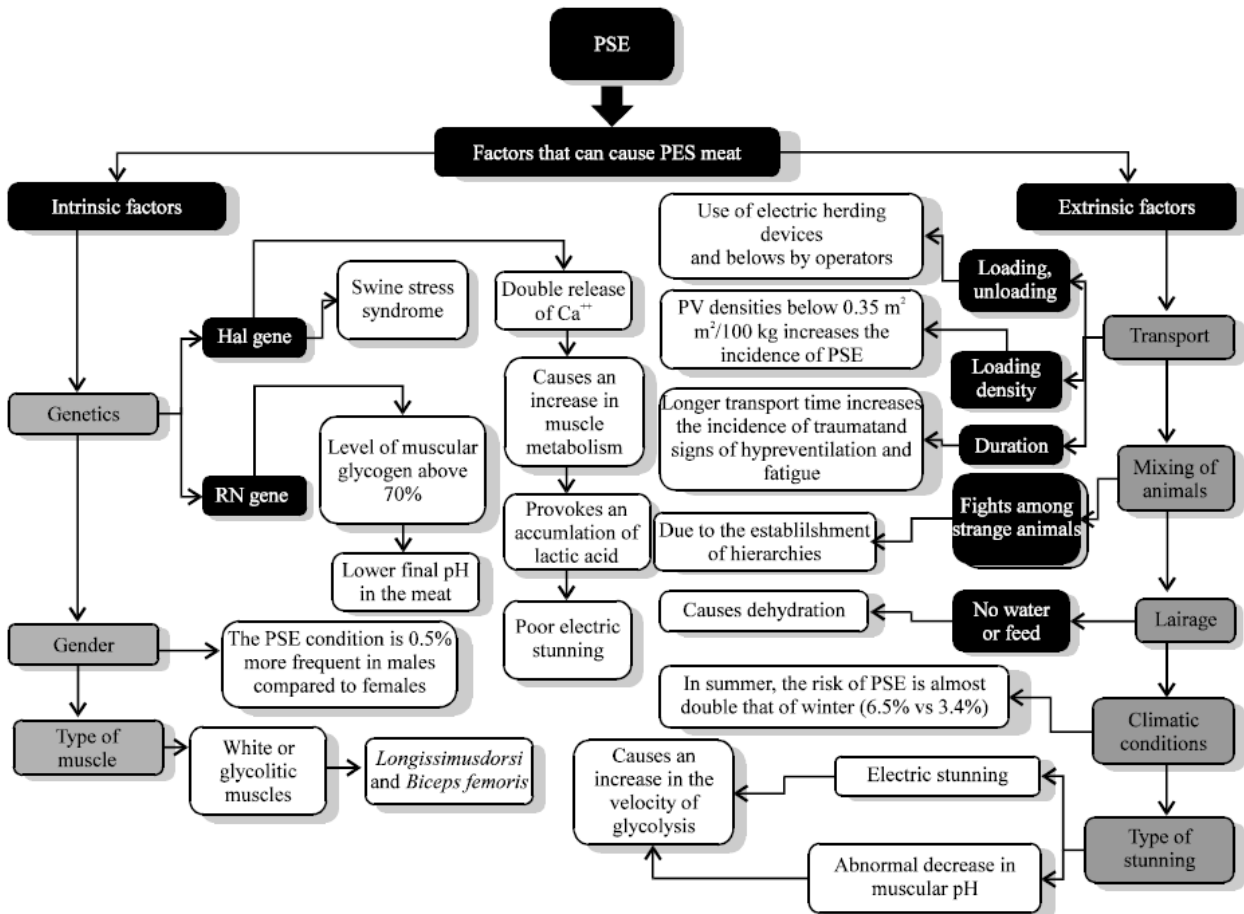


Fig. 1.7 Factors associated with the appearance of PSE in pig (Martinez-Rodriguez et al., 2011)

Acidification in muscle post-mortem is caused by the anaerobic breakdown of glycogen. In PSE meat, the rate of acidification after slaughter is stimulated faster than normal and lower pH values are reached in the muscle when the temperature of the carcass is still high. The combination of low pH and high temperature in PSE meat causes the

denaturation of some muscle proteins resulting in reduction in their WHC. Shrinkage of the myofibrillar components expels the resultant fluid into the extracellular space (between the muscle fibers). When the muscle is cut the extra fluid is released leading to the exudates (drip or purge). The large amount of exudate reflects a poorer WHC as found in PSE meats (Adzitey & Nurul, 2011). The light scattering from meat surface is probably caused by differences in refractive indices of the sarcoplasm and myofibrils. The larger the difference, the higher the scattering and the paler the meat appears. The shrinkage of the myofilament lattice increases the amount of light reflected from the meat. At high scattering the amount of absorbed light is low and the haem pigments (myoglobin) are selectively absorbing green light, thus normal red colour is reduced. This makes PSE meat look less red and more yellow. The low pH in PSE also promotes the oxidation of haem pigments from purple or red myoglobin and oxymyoglobin to brown metmyoglobin (Warriss, 2010).

When animals are exposed to long term stress, e.g. transportation of animals over long distances, long hours of food deprivation, and overcrowding of animals in the lairage over a long period of time before slaughtering, DFD meat can occur. Chronic stress prior to slaughter leads to the depletion of stored glycogen, hence less glycogen is available post-mortem affecting the normal process of acidification and resulting in a high meat pH (Adzitey & Nurul, 2011). The high pH leads to low denaturation of proteins, water is tightly bound and little or no exudate is formed. There is little or no shrinkage of the myofilament lattice and the differences in refractive index of the myofibrils and sarcoplasm are reduced. The muscle absorbs rather than reflects light making the meat appear darker. The closed structure reduces oxygen penetration from the surface and

any oxygen that can reach the interior of muscle is used up by the high cytochrome activity promoted by the high pH (Warriss, 2010).

1.2.1.2.2 Two – toning

Two-toning in meat is the occurrence of both dark and pale colours in the same muscle. In pig carcasses, two-toning is particularly present in the ham. Two-toning in meat may occur if the pH of some parts of the muscle falls faster than normal when the muscle temperature is still high. The inner parts of the muscle tend to cool more slowly so they appear to be paler than the outer parts which cool down more quickly (Warriss, 2010). Similarly, pale beef may occur in the deep muscle of the leg because they cool down more slowly, particularly the inner part of the *Musculus Biceps femoris* (De Boever et al., 2009) and *Musculus Semimembranosus* (Sammel et al., 2002).

1.2.2 Oxidation in meat

Oxidation is a major cause of deterioration of the quality of stored meat and meat products. Oxidative deterioration in meat involves lipid, myoglobin and protein oxidation, leads to a loss of nutritional value and reduced sensory quality (Morrissey et al., 1998; Lund et al., 2011).

1.2.2.1 Lipid oxidation in meat

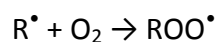
Lipid oxidation is one of the main factors limiting the quality and acceptability of meats and meat products. Damage to lipids may be accentuated in the immediate post-slaughter period and, in particular, during handling, processing, storage and cooking (Morrissey et al., 1998). Lipid oxidation leads to drip losses, off-odour and off-flavour development, colour deterioration, texture defects and nutritional value of meat can be affected (Kanner, 1994; Morrissey et al., 1998).

Lipid oxidation is a free radical chain reaction that is comprised of three primary steps: initiation, propagation, and termination. A three-step simplified free-radical scheme has been postulated as follows:

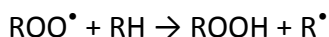
1) Initiation, the first step in lipid oxidation is the removal of hydrogen from a methylene carbon in a fatty acid (RH). This becomes easier as the number of double bonds in the fatty acid increases, which is why polyunsaturated fatty acids are particularly susceptible to oxidation (Halliwell & Chirico, 1993):



2) Propagation, the fatty acyl radical (R^{\bullet}) reacts rapidly with O_2 to form a peroxy radical (ROO^{\bullet}):

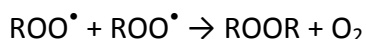
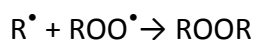
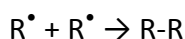


Because ROO^{\bullet} is more highly oxidized than the fatty acyl radical or the fatty acid itself, it will preferentially oxidize other unsaturated fatty acids and propagate the chain reaction:



Hydroperoxides (ROOH) are considered to be the most important initial reaction products that are obtained from lipid oxidation; they are a labile species, of very transitory nature, which undergo changes and deterioration with the radicals. Their breakage causes secondary products such as pentanal, hexanal, 4-hydroxynonenal and malondialdehyde (MDA) (Pearson et al., 1983; Raharjo & Sofos, 1993).

3) Termination, the last step of lipid peroxidation is a termination process in which the ROO^\bullet s react with each other and/or self-destruction to form non-radical products:



Additionally, iron is the most possible catalyst for initiation of lipid peroxidation by catalyzing the generation of most OH^\bullet via Fenton reaction (Min & Ahn, 2005):



Lipid stability in meat and meat products can be influenced by many factors, including species, muscle type, the amount and type of fat in the diet, the nutritional status of the animal at slaughter, the presence or absence of disease or infection, and, increasingly,

the type of processing to which the meat is subjected (mincing, addition of salt, irradiation, refrigeration, freezing and cooking) (Morrissey et al., 1998).

Lipid oxidation measurement techniques

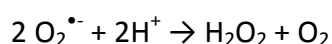
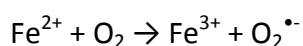
The extent of lipid oxidation can be determined by measuring 1) losses of unsaturated fatty acids, 2) amounts of primary peroxidation products, and 3) amounts of secondary products, such as carbonyls and hydrocarbon gases (Halliwell & Chirico, 1993). Since the primary products of lipid oxidation are hydroperoxides, it is reasonable to determine their concentration as a measure of oxidation. The **peroxide value** test reflects the total concentration of peroxides and hydroperoxides present at a certain time (Kanner & Rosenthal, 1992). Moreover, during the formation of hydroperoxides from unsaturated fatty acids, **conjugated dienes** are typically produced, due to the rearrangement of the double bonds. The resulting conjugated dienes and trienes exhibit an intense absorption at specific wavelengths. Good correlations between conjugated dienes and peroxide value have been found. Ultraviolet detection of conjugated dienes is simple, fast, and requires no chemical reagents and only small amounts of samples are needed. However, this method has less specificity and sensitivity than peroxide value measurements (Shahidi & Zhong, 2005).

Malondialdehyde (MDA) is one of several low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products (Janero, 1990). MDA is formed as a result of the degradation of polyunsaturated fatty acids with 3 or more double bonds (Shahidi & Zhong, 2005). MDA can be determined by

ultraviolet spectrophotometry, HPLC, Gas chromatography and TBA (thiobarbituric acid) test (Fernández et al., 1997). The TBA test is based on the reaction of MDA with TBA to obtain a red pigment, which results from the condensation of two molecules of TBA with one molecule of MDA and the probable elimination of two molecules of water (Sinnhuber et al., 1958). The substances that react with TBA are called TBA-reactive substances (TBARS). Yu and Sinnhuber (1957) reported that MDA was the major TBA reactive substance although later other investigators reported that other oxidation products may also be involved, including α , β -unsaturated aldehydes (e.g. 4-hydroxyalkenals) and several unidentified non-volatile precursors of these substances. Tarladgis et al. (1960) described the distillation method as a technique to measure the MDA content and, later, many modifications and new methods were described.

1.2.2.2 Myoglobin oxidation in meat

As mentioned above, the oxidation of ferrous-myoglobin (Fe^{2+}) to ferric-metmyoglobin (Fe^{3+}) is responsible for discolouration of meat during storage. Ferrous iron (Fe^{2+}) can react with molecular oxygen to produce $\text{O}_2^{\bullet-}$ with concomitant oxidation to ferric iron (Fe^{3+}). The H_2O_2 , which may be produced by dismutation of $\text{O}_2^{\bullet-}$, can react with Fe^{2+} to produce OH^\bullet (Chaijan, 2008). This reaction termed the Fenton reaction is the principal mechanism for myoglobin oxidation as follows:



However, deoxymyoglobin is susceptible to oxidation to form metmyoglobin. The reaction occurs most rapidly at oxygen partial pressures of <7 mm Hg, because at these very low oxygen concentrations, there is not enough oxygen to bind to all the available deoxymyoglobin. Thus, there is plenty of deoxymyoglobin to react with H_2O_2 . At oxygen partial pressures >7 mm Hg, there is more oxygen to bind to deoxymyoglobin; thus, there is less deoxymyoglobin available to react with H_2O_2 . Metmyoglobin reduction influences meat color stability by regenerating ferrous myoglobin. However, this reaction depends on oxygen scavenging, reducing enzymes, and the NADH pool, all of which are limited and continually depleted in post-mortem muscle. Metmyoglobin reduction by endogenous reducing systems in meat may offer a critical strategic approach to decrease metmyoglobin formation and increase fresh meat color stability (AMSA, 2012).

Determination of discolouration in meat is often referred to as the amount of surface area covered by metmyoglobin (Mancini & Hunt, 2005). The percentage of metmyoglobin (%MetMb) formation can be estimated from reflectance values at specific wavelengths (Krzywicki, 1979) and the rate of decrease in a^* (redness) value. A higher a^* value reflects a higher amount of oxymoglobin on the meat surface, and a greater decrease of a^* value with time corresponds with a greater formation of metmyoglobin.

1.2.2.3 Protein oxidation in meat

Protein oxidation (Pox) in muscle food is one of the most innovative issues with increasing interest among meat researchers. Carbonylation is generally recognized as one of the most remarkable chemical modifications in oxidized proteins. The quantification of protein carbonyls by the dinitrophenylhydrazine (DNPH) method is the most common method for assessing Pox in meat systems (Estévez, 2011). Numerous ROS such as $O_2^{\bullet-}$, HO_2^{\bullet} , HO^{\bullet} , H_2O_2 and $ROOH$ are potential initiators of Pox (Butterfield & Stadtman, 1997). Natural components of the muscle tissue such as unsaturated lipids, heme pigments, transition metals and oxidative enzymes are potential precursors or catalysts for the formation of ROS and hence, play a relevant role in the initiation of muscle Pox (Xiong, 2000). It is known that the reaction of radicals with proteins and peptides in the presence of oxygen gives rise to alterations in both the backbone and the amino acid side chains. These oxidative changes include cleavage of peptide bonds, modification of amino acid side chains and formation of covalent intermolecular cross-linked protein derivatives (Lund et al., 2011) as shown in Fig. 1.8.

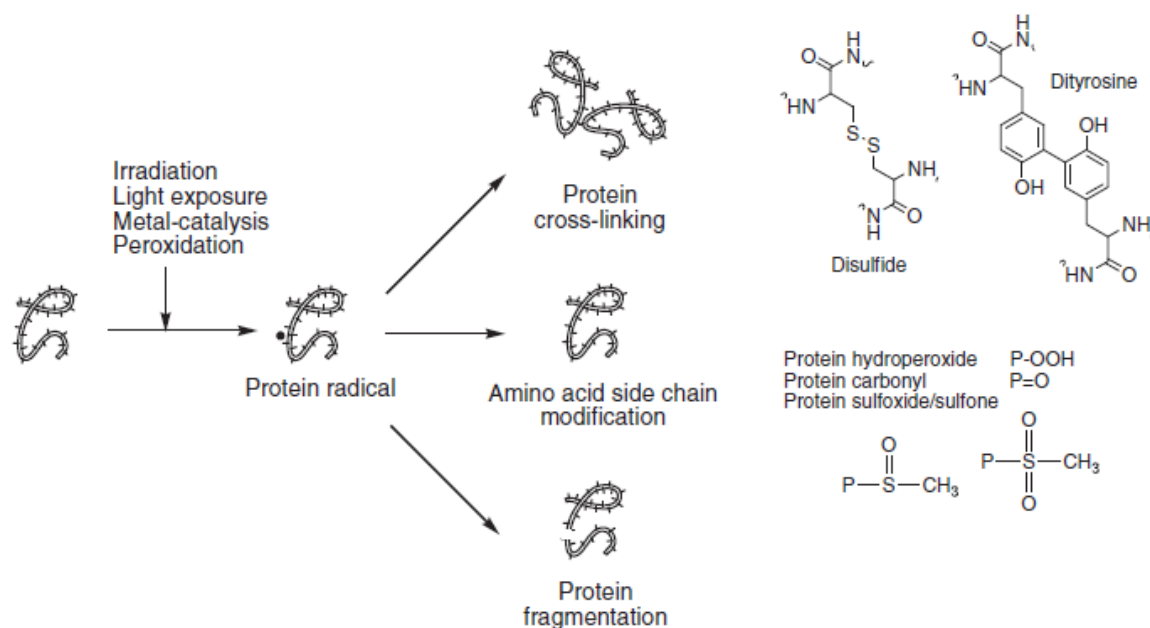
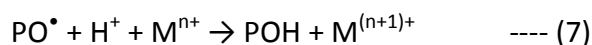
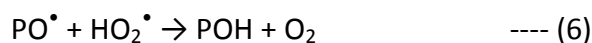
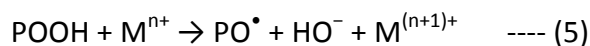
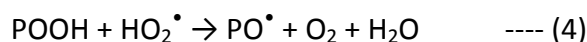
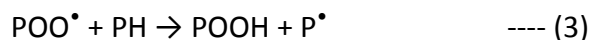


Fig. 1.8 The most common consequences of oxidation of proteins (Lund et al., 2011)

Protein cross-linking has mostly been described as formation of disulfide and dityrosine through the loss of cysteine and tyrosine residues while some of the most general amino acid modifications are the formation of protein carbonyl groups and protein hydroperoxides (Fig. 1.8).

General mechanisms of Pox, as a direct consequence of the abstraction of a hydrogen atom from a susceptible target protein (PH), a carbon-centered protein radical (P^{\bullet}) is formed (Reaction1) (Stadtman & Levine, 2003). The initial P^{\bullet} is consecutively converted into a peroxy radical (POO^{\bullet}) in the presence of oxygen, and to an alkyl peroxide ($POOH$) by abstraction of a hydrogen atom from another susceptible molecule (Reactions 2 and 3). Further reactions with ROS such as the HO_2^{\bullet} radical or with reduced forms of transition metals (M^{n+}) such as Fe^{2+} or Cu^{+} lead to the formation of an alcoxyl radical

(PO[•]) (Reactions 4 and 5) and its hydroxyl derivative (POH) (Reactions 6 and 7) as follows:



The consequences of Pox in meat have been associated with changes in solubility and protein functionality such as gelation and emulsifying properties, the digestibility, or WHC of muscle proteins (Xiong, 2000; Carlin et al., 2006) as well as the post-mortem tenderization of meat are believed to be affected by oxidative reactions (Bertram et al., 2007; Huff-Lonergan et al., 2010; Liu et al., 2010). Moreover, Estévez (2011) has proposed the impact of protein carbonyls on meat quality traits as shown in Fig. 1.9. The precise mechanisms by which protein carbonylation could influence these undesirable Pox-induced changes are not fully clear.

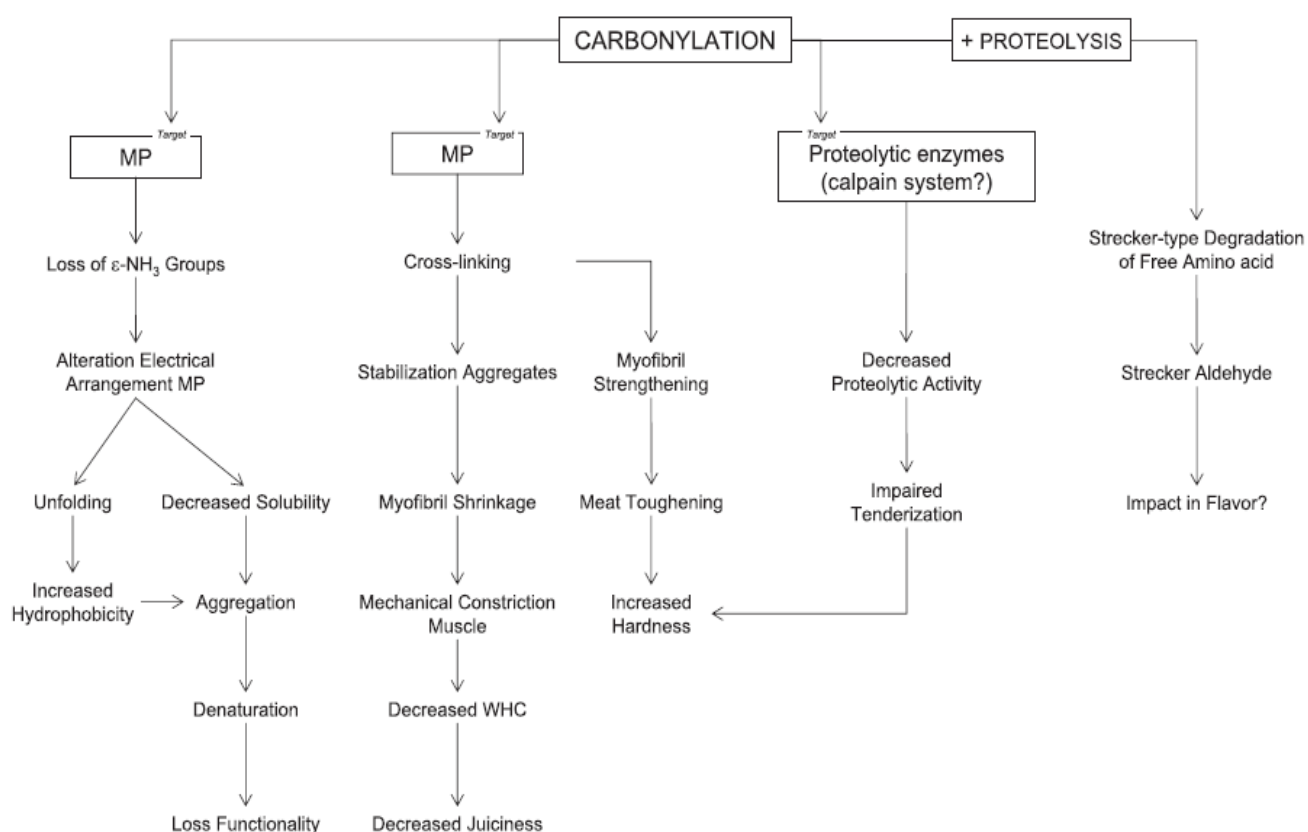


Fig. 1.9 The possible mechanisms by which protein carbonylation could affect technological and sensory traits of meat and meat products (Estévez, 2011)

1.3 ANTIOXIDANT ENZYMES IN MUSCLE POST-MORTEM

As mentioned above, the endogenous antioxidant enzymes play an important role in protecting muscle from ROS during oxidative processes in the living animal. Several studies have shown that antioxidant enzymes present residual activity in muscle post-mortem (Renerre et al., 1996; Pradhan et al., 2000; Chen et al., 2010), but their involvement in slowing down the oxidative processes in meat is largely unknown (Renerre et al., 1996). Chan and Decker (1994) argued that more research was needed to understand the role of skeletal muscle antioxidants under different oxidation

conditions. Moreover, information on factors influencing the activity of antioxidant enzymes in meat is also limited (Renner et al., 1996). It was found that antioxidant enzyme activities differ between meat of different species (Pradhan et al., 2000) and muscle type (Renner et al., 1996; Hernández et al., 2002). Antioxidant enzyme activity could be affected by many post-mortem conditions such as the rate of pH and temperature fall. Therefore, more research on the role of antioxidant enzymes and the effect of some post-mortem conditions is required.

POTENTIAL ROLE OF ANTIOXIDANT ENZYMES AGAINST OXIDATION IN BEEF

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POTENTIAL ROLE OF ANTIOXIDANT ENZYMES AGAINST OXIDATION IN BEEF

ABSTRACT

The objective of this study was to investigate the role of antioxidant enzymes (glutathione peroxidase, GSH-Px; catalase, CAT and superoxide dismutase, SOD) in relation to lipid and protein oxidation in beef through inhibition of their post-mortem activities. Mercaptosuccinate, sodium azide (NaN_3) and diethyldithiocarbamate (DDC) were added to minced beef as specific inhibitors for GSH-Px, CAT and SOD, respectively. The results showed that the activity of GSH-Px was reduced whereas TBARS (lipid oxidation) values and carbonyls (protein oxidation) were increased with increasing concentrations (up to 0.4 mM) of mercaptosuccinate ($P < 0.01$). The CAT activity was completely inhibited and TBARS values were increased at concentrations of 0.03% and 0.06% of NaN_3 compared to lower concentrations ($P < 0.001$) but protein oxidation was not affected ($P > 0.05$). The activity of SOD ($P < 0.05$), TBARS values and protein oxidation ($P < 0.01$) were reduced at the highest concentration of DDC (1 mM). It was concluded that GSH-Px and CAT may have an important role in retarding lipid oxidation in meat post-mortem.

2.1 INTRODUCTION

Oxidative deterioration in meat involves lipid, pigment and protein oxidation, and leads to a loss of nutritional value and reduced sensory quality (Morrissey et al., 1998; Lund et al., 2011). There are several mechanisms to protect muscle *in vivo* and post-mortem against oxidative processes, including the endogenous antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) (Chan & Decker, 1994). SOD is the first line antioxidant enzyme catalysing the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2 . The resultant H_2O_2 is detoxified to O_2 and H_2O by CAT or GSH-Px (Matés et al., 1999).

There are three forms of mammalian SOD (EC 1.15.1.1) according to their location: mitochondrial Mn-SOD with Mn at its active site (Weisiger & Fridovich, 1973), cytosolic CuZn-SOD (McCords & Fridovich, 1969) and extracellular SOD (EC-SOD) (Marklund et al., 1982) containing Cu and Zn at their active site. In skeletal muscle, there is a lower activity of SOD compared to other organs (Marklund, 1984b). Diethyldithiocarbamate (DDC), an effective chelating agent for Cu, has been shown to reversibly inactivate SOD *in vivo* (Heikkila et al., 1976) and *in vitro* (Heikkila et al., 1976, Misra, 1979). It was found that DDC can inhibit both CuZn-SOD and EC-SOD activities in mice (Oury et al., 1992). However, Marklund (1984c) found that EC-SOD is more sensitive to inhibition by DDC than CuZn-SOD *in vitro*. Although it has been reported that DDC inhibits total SOD activities in housefly (Sohal et al., 1984), Iqbal and Whitney (1991) subsequently indicated that DDC treatment did not cause significant loss of Mn-SOD activity *in vitro*. Hence, treating with DDC may provide a tool to investigate the role of SOD in muscle post-mortem.

GSH-Px (EC 1.11.1.19), a selenium-containing enzyme, catalyzes the reduction of H_2O_2 and lipid peroxides to less harmful hydroxides (Matés et al., 1999). The family of GSH-Px comprises four distinct mammalian selenoproteins; 1) the classical GSH-Px which is ubiquitously distributed, 2) the gastrointestinal GSH-Px which is exclusively expressed in the gastrointestinal tract, 3) plasma GSH-Px, which is directed to extracellular compartments and expressed in various tissues in contact with body fluids such as in kidney and 4) phospholipid hydroperoxide GSH-Px, originally assumed to be a universal antioxidant enzyme protecting membrane lipids (Flohé, 1999). A number of mercaptocarboxylic acids and tertiary mercaptans are known to be strong and specific inhibitors of the enzyme GSH-Px. Chaudiere et al. (1984) stated that three of the most potent inhibitors for GSH-Px are mercaptosuccinate, penicillamine, and α -mercaptopropionylglycine. Mercaptosuccinate appears to be the most effective inhibitor in this series and may not affect other biological components to a significant extent.

CAT (EC 1.11.1.6) is a tetrameric enzyme consisting of four identical tetrahedrally arranged subunits of 60 kDa that contains a single ferriprotoporphyrin group per subunit (Matés et al., 1999). CAT protects cells against the damaging effects of H_2O_2 by its active site binding to H_2O_2 and decomposing it to O_2 and H_2O . The activity of CAT can be inhibited by several compounds, such as hydroxylamine (Kulys et al., 2003), 3-Amino-1:2:4-Triazole (Margoliash & Novogrodsky, 1958), cyanide and azide (Blaschko, 1935). Sodium azide (NaN_3) is a very powerful inhibitor of CAT, its action is completely reversible. With a concentration of azide as low as 10^{-6} M, the activity of CAT was inhibited up to 80% *in vitro* from rat liver (Blaschko, 1935).

Activity of these enzymes in muscle post-mortem has been measured in several studies, but their contribution in retarding oxidative deterioration of meat is not well established. Pradhan et al. (2000) demonstrated that CAT seems to play a role in modulating lipid oxidation in post-mortem muscle from different species, however, similar studies have not been performed for SOD and GSH-Px. It may be argued that variation in the activity of these enzymes post-mortem may affect meat oxidative stability. Hence, the objectives of this study were, 1) to determine the effect of DDC, NaN_3 and mercaptosuccinate on the activities of SOD, CAT and GSH-Px, respectively, in minced beef during post-mortem display and, 2) to investigate the effect of this inhibition on lipid, myoglobin and protein oxidation.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of meat samples

Muscles from the forequarter of a young Belgian Blue bull were taken at 48 hours post-mortem, and were stored at $-20\text{ }^{\circ}\text{C}$ until use. The frozen meat was minced and mixed thoroughly with the inhibitor which was dissolved in 0.9% NaCl. The final concentration of inhibitor solution in the meat sample was 2.5%. The specific inhibitors were added separately at three different concentrations versus a control. Samples were prepared in one batch per inhibitor per day. Mercaptosuccinate ($\text{C}_4\text{H}_6\text{O}_4\text{S}$; Sigma-Aldrich) was used as inhibitor for GSH-Px at a final concentration of 0, 0.1, 0.2 and 0.4 mM in meat. Sodium azide (NaN_3 ; Merck), inhibitor for CAT, was used at a final concentration of 0, 0.015, 0.03

and 0.06% in meat. For inhibition of SOD, diethyldithiocarbamate (DDC; $C_5H_{10}NS_2Na$; Merck) was used at a concentration of 0, 0.25, 0.5 and 1 mM in meat.

After mixing meat with the inhibitor solution, 50 g meat patties were made. For each concentration of inhibitor, 6 patties of meat were prepared for sampling at 3 time points during display with 2 replicates per time point. The patties were wrapped with oxygen permeable foil and displayed at 4 °C under fluorescent light (approximately 1200 lux). Samples were removed for analysis after 0, 6 and 12 days of display.

2.2.2 Antioxidant enzyme activity assays

During these analyses, the muscle samples were kept on ice. A 5 g sample was homogenized in 10 ml of 0.05 M phosphate buffer (pH 7.0) and centrifuged at 4 °C for 20 min at 7000g. The supernatant fraction was filtered through glass wool before determining enzyme activities.

The activity of GSH-Px was determined by measuring the oxidation of NADPH. One unit of GSH-Px activity was defined as the amount of extract required to oxidize 1 μ mol of NADPH per min at 25 °C (Hernández et al., 2004). The CAT activity was determined according to the method of Aebi (1983). One unit of CAT activity was defined as the amount of sample required to decompose 1 μ mol of H_2O_2 per min at room temperature. The SOD activity assay was performed as described by Marklund and Marklund (1974) by measuring the inhibition of pyrogallol autoxidation. One unit of enzyme activity was defined as the amount of sample needed to inhibit the reaction by 50%.

2.2.3 Thiobarbituric acid reactive substances

Lipid oxidation was assessed spectrophotometrically by the thiobarbituric acid reactive substances (TBARS) method based on Tarladgis et al. (1960) and is expressed as μg malondialdehyde (MDA) per g meat.

2.2.4 Protein carbonyls

Protein oxidation was assessed by determining the carbonyl content of the samples according to the method of Ganhão et al. (2010). Protein carbonyls were measured following their covalent reaction with 2,4-dinitrophenylhydrazine (DNPH). This reaction leads to the formation of a stable 2,4-dinitrophenylhydrazone product, which is quantified spectrophotometrically at 370 nm, using a molar absorption coefficient of 21.0/ (mM.cm). The total carbonyl content is expressed as nmol DNPH incorporated/mg protein by using the formula (Jongberg et al., 2011).

$$\frac{C_{\text{hydrazone}}}{C_{\text{protein}}} = \frac{A_{370}}{\epsilon_{\text{hydrazone},370} \cdot (A_{280} - A_{370} \cdot 0.43)} \cdot 10^6$$

Where, $\epsilon_{\text{hydrazone}, 370}$ is $21000 \text{ M}^{-1}\text{cm}^{-1}$, and $0.43 = \epsilon_{\text{hydrazone}, 280} / \epsilon_{\text{hydrazone}, 370}$.

2.2.5 Colour measurements

Meat colour L^* , a^* and b^* values were determined by 3 replicate measurements per time point with a Hunterlab Miniscan colour meter (D65 light source, 10° standard

observer, 45°/0° geometry, 1-inch light surface, white standard) after 0, 1, 2, 5, 6, 7, 8, 9 and 12 days of display (AMSA, 2012).

2.2.6 Statistical analysis

Data in the tables and text are presented as mean values \pm standard deviation. Enzyme activities, TBARS values and protein carbonyls were analyzed using a model with the fixed effects of inhibitor, time of display and their interaction term. The colour parameters were analyzed per time point separately using a model with the fixed effect of inhibitor only. The effect of time of display on the enzyme activities was analyzed after grouping the control treatments of the three inhibitor experiments. The General Linear Model (GLM) procedure of SAS[®] Enterprise Guide[®], version 4.3 (SAS Institute Inc., Cary, NC, USA) was used for the statistical analyses. Post-hoc tests were performed at a significance level of $P < 0.05$ using the Tukey correction for multiple tests.

2.3 RESULTS AND DISCUSSION

2.3.1 Time effects on antioxidant enzymes

The control groups of the three inhibitor experiments allowed us to evaluate the effect of time of display on the antioxidant enzyme activities. There were some differences between the different batches prepared for the three inhibitor experiments, but across the control groups following observations could be made. The activity of GSH-Px decreased in time in the order day 0 > day 12 > day 6 ($P < 0.001$). CAT and SOD activities

were lower at day 6 and 12 of display compared to day 0 ($P<0.001$), with no difference between day 6 and 12 of display. Renerre et al. (1996) reported that GSH-Px and SOD activities in different beef muscles decreased until day 6 post-mortem. However, these authors did not find a decrease in CAT activity with time. Also Pradhan et al. (2000) reported that CAT activity in beef was stable during 6 days of cold storage, which is in contrast to our findings.

2.3.2 Effects of mercaptosuccinate

The activity of GSH-Px decreased with increasing concentrations of mercaptosuccinate at the three time points ($P<0.001$) (Table 2.1). The inhibition relative to the control was 17%, 27% and 42% at 0.1, 0.2 and 0.4 mM, respectively. This effect is in line with Chaudiere et al. (1984) who found that mercaptosuccinate inhibited GSH-Px activity in hamster liver, and this inhibition was not pH-dependent around neutral pH. Their results also pointed to the formation of reversible enzyme-inhibitor complexes. The active site selenium is trapped by the rapid binding of the inhibitor in competition with GSH. At day 0, 6 and 12 of display, mercaptosuccinate had no effect on CAT and SOD activities ($P>0.05$). Mercaptosuccinate thus appears to be a specific inhibitor of GSH-Px in muscle.

As expected, TBARS values increased with time of display and were higher at day 6 and 12 compared to day 0 ($P<0.001$; Table 2.1). In line with the reduction in GSH-Px activity, TBARS values increased with increasing concentrations of mercaptosuccinate ($P<0.001$). After 6 days of display, TBARS values were 10%, 23% and 46% higher for the 0.1, 0.2 and 0.4 mM inhibitor treatment respectively compared to the control.

Determination of the protein carbonyl content by the DNPH method is the most common approach followed to assess protein oxidation in meat and meat products (Estévez, 2011). Carbonylation of specific amino acids modifies the conformation of myofibrillar proteins, leading to loss of functionality with a potential impact on several meat quality traits. Protein oxidation is initiated by several reactive oxygen species and is affected by the same factors that also influence lipid oxidation. In the present study, the protein carbonyl content increased 2 to 5-fold between day 0 and day 12 of display ($P<0.001$), in line with several studies that reported an increase in carbonyl content during chill storage in beef (Rowe et al., 2004; Lindahl et al., 2010), pork (Lund et al., 2007a) and turkey (Mercier et al., 1998). There was no significant effect of inhibitor treatment on carbonyl content at day 0 and 6 of storage. However, at day 12 of display, the protein carbonyl content was higher for the mercaptosuccinate treatments compared to the control treatment ($P<0.01$), in line with the increased TBARS values and the decreased GSH-Px activity. Thus, protein and lipid oxidation seemed to be related in the present study, and affected by GSH-Px activity. A possible involvement of GSH-Px in protection against protein oxidation was not investigated before to our knowledge, but this is likely given the similar mechanisms involved in protein and lipid oxidation and the mode of action of GSH-Px. Relationships between lipid and protein oxidation have been observed in several studies, e.g. for turkey meat (Mercier et al., 1998), beef (Mercier et al., 1995) and fish (Srinivasan & Hultin, 1995). GSH-Px reduces non-radical species such as hydrogen peroxide and hydroperoxides, and these are considered as potential initiators of protein oxidation (Estévez, 2011).

The evolution of the colour L^* and a^* values during display is shown in Figure 2.1 and 2.2, respectively. The L^* value strongly decreased during the first day of display and increased slightly during further display. The addition of mercaptosuccinate to the minced beef resulted in higher L^* values compared to the control until day 8 of display ($P < 0.05$; except for day 1). The a^* value was not different between treatments at day 0 and 1 of display and strongly decreased during the first day of display. The a^* values further decreased during display and were slightly but significantly lower for the mercaptosuccinate treatments compared to the control between day 2 and 7 of display ($P < 0.01$). The rate of decrease in a^* value and the formation of metmyoglobin during display are considered good measurements of pigment oxidation. The discolouration of meat from red to brown during cold storage is induced by oxidation of oxymyoglobin (bright cherry red colour) to metmyoglobin (brown colour) (AMSA, 2012). It is well known that a higher a^* (redness) value reflects a higher content of oxymyoglobin on the meat surface. Therefore, a higher rate of decrease in a^* value corresponds to an increase in metmyoglobin formation on the meat surface. In this study the difference in decline in the a^* value suggests that GSH-Px is also involved in reducing myoglobin oxidation parallel to its effects on lipid oxidation, at least during the initial period of display. However, the colour data have to be interpreted carefully since the addition of the inhibitor appeared to affect the colour lightness.

Table 2.1 Effect of Mercaptosuccinate on GSH-Px, CAT, and SOD activities, TBARS values and protein carbonyls in minced beef samples during display (mean \pm SD; n=2)

		Mercaptosuccinate concentration			
	Day	Control	0.1 mM	0.2 mM	0.4 mM
GSH-Px (U/g)	0	1.15 \pm 0.05 ^{a,x}	0.94 \pm 0.02 ^{b,x}	0.85 \pm 0.01 ^{bc,x}	0.72 \pm 0.04 ^{c,x}
	6	0.86 \pm 0.03 ^{a,y}	0.78 \pm 0.01 ^{ab,y}	0.73 \pm 0.00 ^{bc,xy}	0.62 \pm 0.01 ^{c,x}
	12	1.08 \pm 0.13 ^{a,x}	0.84 \pm 0.08 ^{b,xy}	0.69 \pm 0.07 ^{c,y}	0.48 \pm 0.04 ^{d,y}
$P_{\text{treatment}} < 0.001$; $P_{\text{time}} < 0.001$; $P_{\text{treatment} \times \text{time}} < 0.001$					
CAT (U/g)	0	138.01 \pm 8.23 ^x	130.73 \pm 10.77 ^x	136.65 \pm 9.94 ^x	134.25 \pm 7.44 ^x
	6	77.01 \pm 10.42 ^y	77.57 \pm 5.13 ^z	74.57 \pm 2.80 ^z	77.35 \pm 7.41 ^z
	12	90.32 \pm 3.07 ^y	105.77 \pm 1.07 ^y	92.59 \pm 7.27 ^y	107.16 \pm 4.36 ^y
$P_{\text{treatment}} = 0.285$; $P_{\text{time}} < 0.001$; $P_{\text{treatment} \times \text{time}} = 0.025$					
SOD (U/g)	0	59.20 \pm 2.02 ^x	63.10 \pm 1.28 ^x	61.17 \pm 0.94 ^x	62.51 \pm 3.53 ^x
	6	40.39 \pm 0.25 ^y	40.41 \pm 1.24 ^y	41.65 \pm 3.76 ^y	46.47 \pm 3.36 ^y
	12	39.49 \pm 0.47 ^y	40.54 \pm 0.38 ^y	39.05 \pm 0.35 ^y	41.15 \pm 0.29 ^y
$P_{\text{treatment}} = 0.041$; $P_{\text{time}} < 0.001$; $P_{\text{treatment} \times \text{time}} = 0.344$					
TBARS (μ g/g)	0	0.34 \pm 0.13 ^y	0.35 \pm 0.02 ^z	0.43 \pm 0.04 ^z	0.57 \pm 0.02 ^y
	6	6.37 \pm 0.09 ^{d,x}	7.04 \pm 0.44 ^{c,y}	7.81 \pm 0.27 ^{b,y}	9.27 \pm 0.40 ^{a,x}
	12	6.33 \pm 0.19 ^{c,x}	7.69 \pm 0.10 ^{b,x}	8.64 \pm 0.24 ^{a,x}	9.06 \pm 0.26 ^{a,x}
$P_{\text{treatment}} < 0.001$; $P_{\text{time}} < 0.001$; $P_{\text{treatment} \times \text{time}} < 0.001$					
Protein carbonyls (nmol/mg protein)	0	5.11 \pm 0.42	4.51 \pm 0.71 ^y	4.65 \pm 0.00 ^y	4.49 \pm 0.88 ^y
	6	7.66 \pm 0.73	8.50 \pm 0.54 ^y	9.64 \pm 1.52 ^y	7.87 \pm 2.04 ^y
	12	10.85 \pm 3.62 ^b	17.87 \pm 0.67 ^{a,x}	18.84 \pm 3.22 ^{a,x}	23.18 \pm 1.13 ^{a,x}
$P_{\text{treatment}} = 0.008$; $P_{\text{time}} < 0.001$; $P_{\text{treatment} \times \text{time}} = 0.003$					

^{a,b,c,d} Within a row, mean values with different superscripts differ significantly at $P < 0.05$

^{x,y,z} Within a column and variable, mean values with different superscripts differ significantly at $P < 0.05$

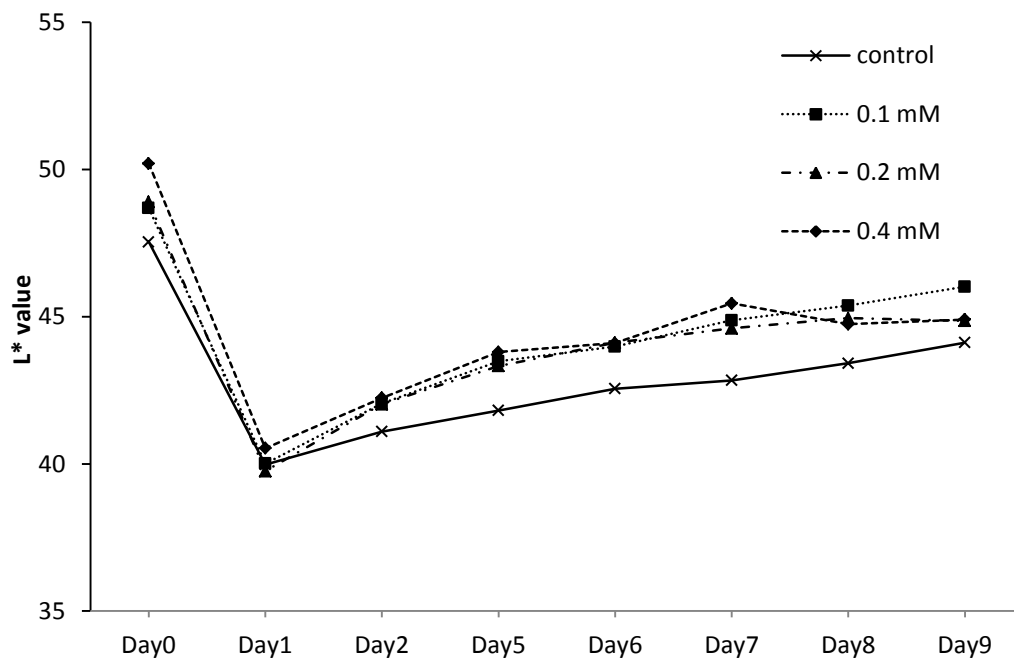


Fig. 2.1 Effect of Mercaptosuccinate at 0, 0.1, 0.2 and 0.4 mM on colour L^* value in minced beef samples during display

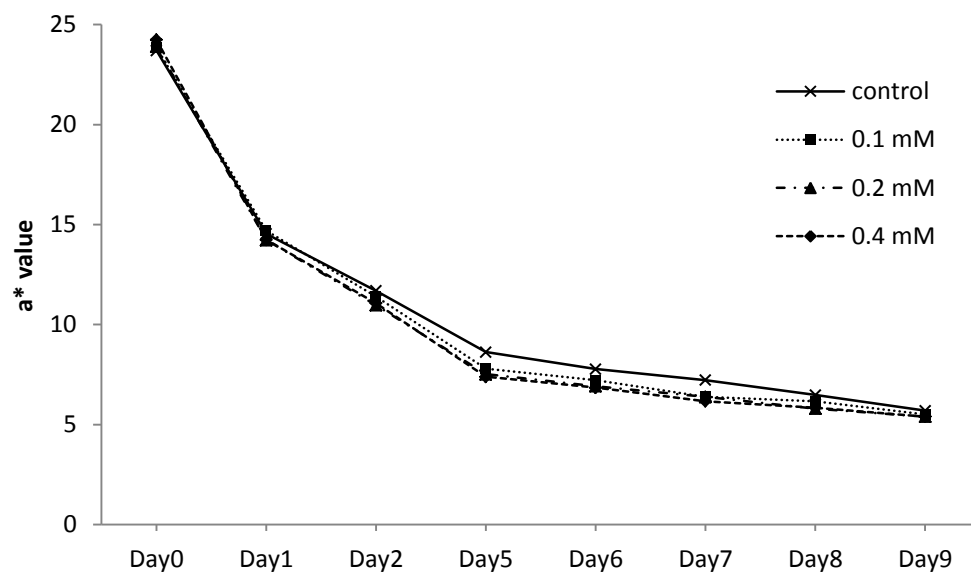


Fig. 2.2 Effect of Mercaptosuccinate at 0, 0.1, 0.2 and 0.4 mM on colour a^* value in minced beef samples during display

2.3.3 Effects of NaN_3

The CAT activity in the minced meat samples was significantly reduced at the concentration of 0.015% NaN_3 when compared to the control and complete inhibition was observed at the concentration of 0.03% and 0.06% NaN_3 ($P < 0.001$; Table 2.2). This result supports the work of Pradhan et al. (2000) who reported that there was no discernible CAT activity in beef *M. Semimembranosus* (SM) samples treated with NaN_3 at 0.03% in meat. It is known that NaN_3 inhibits CAT by reacting with the heme prosthetic group of the enzyme (White et al., 1964).

At day 0, 6 and 12 of display, there was no significant difference in SOD activity in all concentrations of NaN_3 ($P > 0.05$). The effect of NaN_3 on GSH-Px activity seemed to be inconsistent. GSH-Px activity at 0.03% NaN_3 was significantly lower at day 0 and 12 of display, but higher at day 6 of display ($P < 0.001$) compared to the other concentrations. Hence, it may be concluded that NaN_3 is a specific inhibitor of CAT activity.

Similar to the results from the mercaptosuccinate treatment, TBARS values increased with time of display and were thus higher at day 6 and 12 than day 0 of display ($P < 0.001$). Interestingly, the TBARS values increased with decreasing activity of CAT and increasing concentration of NaN_3 at day 6 and 12 of display ($P < 0.001$; Table 2.2). After 6 days of display, TBARS values were 87%, 158% and 134% higher in the 0.015%, 0.03% and 0.06% NaN_3 treatments compared to the control, respectively, while the relative inhibition of CAT was 73%, 100% and 100%, respectively. Similarly, Pradhan et al. (2000) demonstrated that when 0.03% NaN_3 was used for inhibiting CAT in ground beef SM, lipid oxidation (as measured by peroxide values) was significantly higher in treated than in control samples by 43% and 55% for day 2 and day 4, respectively, of cold storage.

Our result supports that CAT is involved in protecting muscle from lipid oxidation post-mortem.

As for the mercaptosuccinate inhibitor experiment, the protein carbonyl content significantly increased with time of display ($P < 0.001$), but there was no effect of NaN_3 treatment ($P > 0.05$). In this experiment, lipid oxidation seemed to be affected by the reduction in CAT activity following the addition of NaN_3 , whereas protein oxidation was not. Although several studies reported an association between lipid and protein oxidation as mentioned above, this was not observed in all studies. E.g. Lund et al. (2007b) found that the protein carbonyl content in beef patties did not differ among antioxidant treatments and different packaging atmospheres after 1 day of storage, but the presence of oxygen in the packaging atmospheres increased lipid oxidation markedly after 1 day of storage.

The colour L^* value was strongly affected by the addition of NaN_3 (data not shown), hence the effects of CAT on colour stability could not be assessed properly and are therefore not discussed here. Since NaN_3 inhibits CAT by reacting with the heme prosthetic group, it could however be hypothesised that it also inhibits the pro-oxidant activity of the heme pigments as it binds to their heme group (Pradhan et al., 2000).

Table 2.2 Effect of Sodium azide (NaN₃) on CAT, SOD, and GSH-Px activities, TBARS values and protein carbonyls in minced beef samples during display (mean \pm SD; n=2)

		NaN ₃ concentration			
	Day	Control	0.015%	0.03%	0.06%
CAT	0	92.30 \pm 16.79 ^a	27.25 \pm 8.07 ^b	ND	ND
(U/g)	6	85.60 \pm 4.88 ^a	20.43 \pm 11.58 ^b	ND	ND
	12	86.77 \pm 5.04 ^a	17.49 \pm 3.66 ^b	ND	ND
P _{treatment} <0.001; P _{time} =0.228; P _{treatment x time} =0.729					
SOD	0	52.56 \pm 3.37 ^x	47.17 \pm 0.13	44.35 \pm 0.53	49.34 \pm 5.60 ^x
(U/g)	6	44.38 \pm 1.92 ^{xy}	36.42 \pm 0.58	37.66 \pm 1.92	38.72 \pm 0.70 ^{xy}
	12	37.26 \pm 9.28 ^y	35.36 \pm 1.65	31.47 \pm 1.02	35.03 \pm 0.92 ^y
P _{treatment} =0.027; P _{time} <0.001; P _{treatment x time} =0.874					
GSH-Px	0	1.25 \pm 0.08 ^{a,x}	1.30 \pm 0.02 ^{a,x}	1.15 \pm 0.02 ^{b,x}	1.22 \pm 0.03 ^{ab,x}
(U/g)	6	0.95 \pm 0.01 ^{ab,y}	0.93 \pm 0.02 ^{ab,y}	1.00 \pm 0.03 ^{a,y}	0.91 \pm 0.01 ^{b,y}
	12	0.94 \pm 0.03 ^{a,y}	0.92 \pm 0.03 ^{ab,y}	0.85 \pm 0.01 ^{b,z}	0.92 \pm 0.03 ^{ab,y}
P _{treatment} =0.001; P _{time} <0.001; P _{treatment x time} <0.001					
TBARS	0	0.08 \pm 0.01 ^y	0.14 \pm 0.02 ^z	0.16 \pm 0.02 ^z	0.18 \pm 0.01 ^z
(μ g/g)	6	3.02 \pm 0.77 ^{c,x}	5.66 \pm 0.40 ^{b,y}	7.84 \pm 0.40 ^{a,y}	7.07 \pm 0.17 ^{a,y}
	12	3.78 \pm 0.20 ^{c,x}	7.95 \pm 0.36 ^{b,x}	9.76 \pm 0.36 ^{a,x}	9.15 \pm 0.69 ^{a,x}
P _{treatment} <0.001; P _{time} <0.001; P _{treatment x time} <0.001					
Protein carbonyls	0	3.96 \pm 0.04	4.26 \pm 0.67 ^y	5.00 \pm 0.45	5.91 \pm 1.51
(nmol/mg protein)	6	6.56 \pm 0.42	7.83 \pm 1.12 ^{xy}	6.82 \pm 0.81	6.44 \pm 1.36
	12	9.11 \pm 0.93	10.27 \pm 2.61 ^x	10.28 \pm 0.36	10.78 \pm 2.93
P _{treatment} =0.523; P _{time} <0.001; P _{treatment x time} =0.825					

^{a,b,c,d} Within a row, mean values with different superscripts differ significantly at P<0.05.

^{x,y,z} Within a column and variable, mean values with different superscripts differ significantly at P<0.05.

ND: Not detected

2.3.4 Effects of DDC

There was a significant effect of the inhibitor DDC on the activity of SOD ($P < 0.002$; Table 2.3). At the three times of display, the highest concentration of inhibitor (1 mM) resulted in clearly lower values for the SOD activity compared to the control and the other concentrations, although the differences did not reveal significant according to the post-hoc test. Across times of display, the mean SOD activity at 1mM DDC was 17% lower compared to the control. There were no differences in SOD activity between the control and 0.25 and 0.5 mM treatments. Dumay et al. (2006) reported that 0.5 mM DDC efficiently inhibited SOD activity (more than 80%) in HeLa cells. Similarly, Heikkila et al. (1976) demonstrated that incubating pure SOD or homogenates of brain or liver with 1 mM DDC for 1.5 hours resulted in total loss of SOD activity. When injecting 1.5 g of DDC/kg into mice, the total SOD activity at 3 hours post injection was decreased by 86%, 71%, and 48% in whole blood, liver, and brain, respectively. *In vivo*, Sohal et al. (1984) found that total SOD activity decreased 20% and 34% when 1 mM and 10 mM DDC was administered to the housefly. From this study, CuZn-SOD activity showed 15% and 59% reduction in response to 1 mM and 10 mM DDC administration, respectively and Mn-SOD also showed a reduction in the DDC-administered groups but less drastic than CuZn-SOD activity. The possible mechanism of inhibition is DDC binding to Cu at the active site of CuZn-SOD, resulting in elimination of the catalytic activity. The reaction is a relatively slow reaction which apparently leads to a cooperative binding of two DDC molecules per Cu atom with consequent removal of the metal from the protein (Cocco et al., 1981).

Table 2.3 Effect of Diethyldithiocarbamate (DDC) on SOD, CAT, and GSH-Px activities, TBARS values and protein carbonyls in minced beef samples during display (mean \pm SD; n=2)

		DDC concentration			
	day	Control	0.25 mM	0.5 mM	1 mM
SOD	0	50.95 \pm 5.52 ^x	51.00 \pm 1.54 ^x	50.28 \pm 6.54 ^x	43.70 \pm 0.77 ^x
(U/g)	6	33.49 \pm 0.26 ^y	34.98 \pm 1.51 ^y	32.93 \pm 0.21 ^y	27.56 \pm 1.21 ^y
	12	42.43 \pm 0.13 ^{xy}	39.53 \pm 1.54 ^y	36.12 \pm 2.98 ^y	32.85 \pm 2.49 ^{xy}
P _{treatment} =0.002; P _{time} <0.001; P _{treatment x time} =0.802					
CAT	0	154.78 \pm 2.65 ^{a,x}	152.50 \pm 2.22 ^{ab,x}	139.14 \pm 6.68 ^{b,x}	141.86 \pm 3.41 ^{ab,x}
(U/g)	6	127.09 \pm 6.66 ^y	129.06 \pm 10.25 ^y	130.87 \pm 3.93 ^x	125.49 \pm 6.55 ^y
	12	120.26 \pm 7.94 ^{a,y}	110.62 \pm 7.53 ^{ab,z}	106.69 \pm 3.77 ^{ab,y}	104.34 \pm 6.30 ^{b,z}
P _{treatment} <0.001; P _{time} <0.001; P _{treatment x time} =0.033					
GSH-Px	0	1.20 \pm 0.01 ^{b,x}	1.21 \pm 0.05 ^{ab,x}	1.25 \pm 0.04 ^{ab,x}	1.34 \pm 0.06 ^{a,x}
(U/g)	6	0.96 \pm 0.05 ^y	1.02 \pm 0.02 ^y	1.05 \pm 0.02 ^y	1.05 \pm 0.03 ^y
	12	1.19 \pm 0.10 ^{a,x}	1.19 \pm 0.08 ^{a,x}	1.04 \pm 0.08 ^{b,y}	1.05 \pm 0.04 ^{b,y}
P _{treatment} =0.326; P _{time} <0.001; P _{treatment x time} <0.001					
TBARS	0	0.34 \pm 0.05 ^z	0.25 \pm 0.05 ^z	0.20 \pm 0.06 ^z	0.11 \pm 0.04 ^z
(μ g/g)	6	4.32 \pm 0.34 ^{a,y}	4.28 \pm 0.29 ^{a,y}	4.04 \pm 0.10 ^{a,y}	3.34 \pm 0.05 ^{b,y}
	12	5.37 \pm 0.36 ^{b,x}	5.95 \pm 0.03 ^{a,x}	5.73 \pm 0.17 ^{a,x}	5.10 \pm 0.08 ^{b,x}
P _{treatment} <0.001; P _{time} <0.001; P _{treatment x time} <0.001					
Protein carbonyls	0	3.79 \pm 0.52 ^y	4.64 \pm 0.53	3.03 \pm 0.78 ^y	4.09 \pm 0.09 ^y
(nmol/mg protein)	6	8.11 \pm 0.69 ^x	5.24 \pm 0.90	5.00 \pm 0.04 ^{xy}	6.06 \pm 0.79 ^{xy}
	12	10.73 \pm 1.72 ^{a,x}	7.51 \pm 0.46 ^b	7.78 \pm 1.11 ^{ab,x}	7.34 \pm 0.23 ^{b,x}
P _{treatment} =0.002; P _{time} <0.001; P _{treatment x time} =0.037					

^{a,b,c,d} Within a row, mean values with different superscripts differ significantly at P<0.05

^{x,y,z} Within a column and variable, mean values with different superscripts differ significantly at P<0.05

For CAT activity, there were no significant differences between the treatments at day 6 of display, whereas 0.5 mM DDC at day 0 of display and 1 mM DDC at day 12 of display reduced the activity of CAT compared to the other treatments ($P < 0.001$). This corresponds to Sohal et al. (1984) who observed a 12% reduction of CAT activity by administering 10 mM DDC to a housefly compared to the control. DDC can thus not be considered a specific inhibitor for SOD only. At day 0 of display, the GSH-Px activity from the 1 mM DDC treatment was significantly higher than for the other concentrations. In contrast, at day 12 of display, the GSH-Px activity from the 0.5 and 1 mM DDC treatments were significantly lower than control and 0.25 mM DDC. At day 6 of display, there was no significant difference in GSH-Px activity between the treatments. The effect of DDC on GSH-Px activity is thus inconsistent.

Surprisingly, TBARS values at day 6 and 12 of display decreased with increasing concentration of DDC up to 1 mM or lower activity of SOD in beef ($P < 0.001$). This result suggests that SOD is not involved in reducing lipid oxidation in meat post-mortem. The reason for this unexpected opposite finding is unclear. Marklund (1984b) showed that SOD was much lower in muscle than in other organs of cattle, thus SOD might be less important for retarding lipid oxidation in muscle than in other organelles. During the display period, TBARS values were higher at day 6 and 12 of display compared to day 0 ($P < 0.001$), like for the other inhibitor experiments.

Protein carbonyls displayed a similar trend as TBARS in this inhibitor experiment. The protein carbonyl content significantly increased with time of display ($P < 0.001$), and was lower for the 1 mM DDC treatment at day 12 of display compared to the control treatment. This observation confirms the relationship between lipid and protein

oxidation found in several studies, as discussed above. In muscle, however, both processes seem not to be inhibited by SOD activity.

Similarly as for NaN_3 , DDC interfered with the meat lightness so that the effects of SOD on colour stability could not be assessed in this study.

2.4 CONCLUSION

Making use of specific inhibitors of activity is a tool to study the role of enzymes. In this study, it was concluded that the post-mortem activities of GSH-Px and CAT in meat can be specifically inhibited by mercaptosuccinate and sodium azide respectively. The SOD activity was inhibited by DDC, but this inhibition was not specific. Inhibiting the GSH-Px and CAT activities concomitantly increased lipid oxidation and inhibiting the GSH-Px activity also increased protein oxidation and the initial formation of metmyoglobin. Inhibition of SOD unexpectedly decreased lipid and protein oxidation in meat. This suggests that GSH-Px and CAT have an important role in retarding oxidation, while SOD is probably not involved in protecting fresh meat against oxidation during storage. More research is warranted to estimate the contribution of the post-mortem activity of these endogenous enzymes in protecting meat against oxidative processes relative to other antioxidants, and to examine sources of variation in these enzyme activities, e.g. breed of animal, type of muscle, slaughter process and other factors.

**EFFECT OF MUSCLE AND POST–MORTEM RATE OF PH AND TEMPERATURE
FALL ON ANTIOXIDANT ENZYME ACTIVITIES IN BEEF**

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EFFECT OF MUSCLE AND POST-MORTEM RATE OF PH AND TEMPERATURE FALL ON ANTIOXIDANT ENZYME ACTIVITIES IN BEEF

ABSTRACT

The aim of this study was to investigate the effect of muscle, inner and outer *M. Biceps femoris* (IBF and OBF respectively) and *M. Longissimus dorsi* (LD), on the post-mortem rate of pH and temperature fall, and the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) during simulated retail display. At day 0 of display (2 days post-mortem), the CAT and GSH-Px activities were lower in IBF than in OBF and LD ($P<0.001$), and the SOD activity was lower in OBF compared to IBF and LD ($P<0.001$). At day 10 of display, SOD and CAT activities had decreased in all three muscles compared to day 0 ($P<0.001$), whereas the GSH-Px activity did increase with time of display. Across muscles, there were significant relationships between temperature fall, colour, lipid and colour stability and antioxidant enzyme activities.

3.1 INTRODUCTION

It is well known that the post-mortem temperature evolution and the rate and extent of pH fall have a considerable effect on several meat quality attributes in different animal species. Evidently, variation in the rate and extent of glycolysis is related to variation in the post-mortem activity of enzymes involved (Scheffler et al., 2011). Conversely, this may affect the activity of enzymes in other pathways. In beef, as for pork, a lower pH and higher temperature early post-mortem are associated with paler colour and lower water-holding capacity, although quality defects are much less severe for beef than for pork (Fischer & Hamm, 1980; Warriss & Brown, 1987; Fernández et al., 1994; Steen et al., 1997). Fischer and Hamm (1980) attributed the accelerated breakdown of glycogen in fast-glycolysing bovine *Musculus Psoas major* to an activation of the phosphorylase system. Regarding beef tenderness, the effects of early post-mortem conditions are not unequivocal. O'Halloran et al. (1997) reported that fast glycolysing beef *M. Longissimus* samples from Hereford×Friesian heifers were more tender in sensory analysis compared to slow glycolysing samples, caused by increased proteolysis that was related to an enhanced release of lysosomal cathepsins, increased calpain I activity and decreased calpastatin activity. On the other hand, Steen et al. (1997) found a weak positive relationship in *M. Longissimus* double-muscled Belgian Blue bulls between shear force and temperature at 3 hours post-mortem but not with pH early post-mortem. Several other reports identified an intermediate pH decline (i.e. pH 5.9–6.2 at 1.5 hour post-mortem) as the optimum for obtaining the most tender meat after ageing (Smulders et al., 1990; Hwang & Thomson, 2001a), with significant interactions between pH and temperature at 1.5 hour post-mortem on μ -calpain and calpastatin activities. In pork, a

fast early post-mortem pH decline influences proteolysis of desmin and talin by activating μ -calpain, contributing to the development of PSE meat (Bee et al., 2007). Significant differences between two pig lines, largely differing in pH 1 hour post-mortem, were found for several enzyme activities in *Musculus Transversus abdominis* at 3 hours post-mortem, whereas other enzymes were not affected (Claeys et al., 2001). Relationships across and within lines suggested that increased protein denaturation as a result of stimulated glycolysis negatively affected the activity of several enzymes, such as μ -calpain, m-calpain, pyroglutamyl aminopeptidase I, acid lipase and neutral phospholipase. It thus appears that both an activation or an impairment of enzyme activity may occur with varying post-mortem conditions.

Oxidative deterioration in meat leads to a loss of nutritional value and reduced sensory quality (Daun et al., 2001; Hernández et al., 2004). To minimise oxidation reactions, there are several mechanisms in muscle including the endogenous antioxidant systems α -tocopherol, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). *In vivo*, SOD is the first line antioxidant enzyme defending against reactive oxygen species (ROS) and free radicals, while CAT and GSH-Px reduce hydrogen peroxide and alkyl hydroperoxides in the cytoplasm (Chan & Decker, 1994; Matés et al., 1999; Muller et al., 2007). Some previous studies aimed to correlate the post-mortem activity of these antioxidant enzymes to lipid and myoglobin oxidation in meat (Renner et al., 1996; Chen et al., 2010). However, information on the contribution of these enzymes to retarding the oxidative deterioration of muscle post-mortem is relatively scarce, and particularly studies on the effect of variation in post-mortem rate of glycolysis and temperature fall on these antioxidant enzymes are lacking. It may be hypothesised that

a reduced activity of these enzymes due to increased protein denaturation negatively affects post-mortem muscle oxidative stability.

Double-muscled Belgian blue cattle have a more glycolytic muscle fibre type and heavier carcasses compared to conventional animals resulting in a faster pH decline and slower temperature fall post-mortem compared to carcasses of non double-muscled animals (Cliquart et al., 1998; De Smet, 2004). Particularly in the deeper laying muscles of the hindquarter, this may induce heat shortening and increase protein denaturation, resulting in aberrant meat quality (Sammel et al., 2002; De Boever et al., 2009). The objective of this work was therefore to investigate the effect of muscle type; outer and inner *M. Biceps femoris* (OBF and IBF respectively) and *M. Longissimus dorsi* (LD), and simulated retail display on the activity of the major antioxidant enzymes (GSH-Px, SOD and CAT) in relation to the rate of pH and temperature fall and quality parameters such as colour, lipid oxidation and metmyoglobin formation in double-muscled Belgian blue beef.

3.2 MATERIALS AND METHODS

3.2.1 Animals and meat samples

A total of 40 double-muscled Belgian Blue young bulls with an average age and carcass weight at slaughter of 21.6 ± 2.7 months and 502.4 ± 42.2 kg respectively were used. Animals originated from 16 different farms and were slaughtered in 4 commercial abattoirs on 5 days. Animals were slaughtered and cooled according to common

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practices, i.e. stunned by captive bolt, shackled and bled immediately, dehided, eviscerated, split and sent to a conventional chilling room after approximately 1 hour. At 48 hours post-mortem, a slice of 2.5 cm thickness of the LD was taken at the height of the 7th rib. At the thickest part of the hind leg a slice of 2.5 cm thickness was taken from the *M. Biceps femoris* (BF). The inner and outer BF sample (IBF and OBF) were 6 cm width and were taken from the long head of the BF. The IBF and OBF sample were taken from the most interior and the most exterior part respectively. The middle part was not further used. A first set of subsamples was vacuum packed and frozen at -20°C until enzyme activity and α -tocopherol analysis. For the simulated retail display, a second set of subsamples was wrapped in oxygen permeable foil and continuously displayed at 4°C under fluorescent light (approximately 1200 lux) for 10 days.

3.2.2 Temperature and pH measurements

The temperature and pH were measured at 1, 3, 5 and 48 hour post-mortem in the LD muscle at 8 cm depth and in the IBF at 10 cm depth. The duration of potential heat shortening was calculated based on the definition of $\text{pH} < 6.0$ and temperature $> 35^{\circ}\text{C}$ (Thompson, 2002).

3.2.3 Colour measurements

Beef colour L^* , a^* and b^* values and reflectance values were determined with a Hunterlab Miniscan colour meter (D65 light source, 10° standard observer, $45^{\circ}/0^{\circ}$

geometry, 1-inch light surface, white standard) at 0, 1, 7 and 10 days of display (AMSA, 2012). Chroma was calculated as $(a^{*2}+b^{*2})^{1/2}$. The percentage metmyoglobin (%MetMb) was determined using reflectance values at the wavelengths 520, 530, 570, 580 and 700 nm and using the formulas of Krzywicki (1979), as modified by Lindahl et al. (2001).

3.2.4 Lipid oxidation and protein solubility

Lipid oxidation and protein denaturation were determined on samples at day 0 and day 10 of display. Lipid oxidation was assessed spectrophotometrically by the thiobarbituric acid reactive substances (TBARS) method based on Tarladgis et al. (1960) and is expressed as μg malondialdehyde (MDA) per g meat.

Protein solubility was measured in a low ionic strength solution (150 mM NaCl) according to Claeys et al. (2002), and was expressed in mg protein per g meat. A lower protein solubility was considered to reflect a higher degree of protein denaturation.

3.2.5 Alpha-tocopherol content

For analysis of the α -tocopherol concentration, 2 g of meat was first homogenised in absolute ethanol, kept at 78 °C for 30 min and cooled to room temperature. Then, 17.5% of water was added and the extract was cooled on ice followed by centrifugation to remove the fat. The ethanol extract was subsequently extracted twice with hexane. The hexane was evaporated under nitrogen, and the remaining fraction was dissolved in

methanol and injected on a reversed phase C18 column (Supelcosil LC18, 25 cm×4.6 mm×5 μ m, Sigma-Aldrich, Bornem, Belgium) in an ÄKTA purifier HPLC apparatus (GE Healthcare, Diegem, Belgium). The mobile phase was a mixture of methanol/water (97/3; v/v) and the elution was performed at a flow rate of 2 ml/min. UV-detection was accomplished at a wavelength of 292 nm. The α -tocopherol content of the samples was determined by comparison of the peak areas with those obtained from a standard curve of α -tocopherol. The concentration is expressed as μ g α -tocopherol per g meat.

3.2.6 Antioxidant enzyme activity assays

Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) activities were determined on samples at the start (day 0, equal to day 2 post-mortem) and at the end of display (day 10). After thawing, the muscle samples were kept on ice during the procedure. A 5 g muscle sample was homogenised in 10 ml of 0.05 M phosphate buffer (pH 7.0) and centrifuged at 4 °C for 20 min at 7000g. The supernatant fraction was filtered through glass wool before determining enzyme activities.

The SOD activity assay was performed as described by Marklund and Marklund (1974) by measuring the inhibition of pyrogallol autoxidation. One unit of enzyme activity was defined as the amount of extract needed to inhibit the rate of oxidation by the control (no SOD) by 50%. The activity of GSH-Px was determined by measuring the oxidation of NADPH according to Hernández et al. (2004). One unit of GSH-Px activity was defined as the amount of extract needed to oxidise 1 μ mol of NADPH per min at 25 °C. The CAT

activity was determined according to the method of Aebi (1983). One unit of CAT activity was defined as the amount of extract needed to decompose 1 μmol of H_2O_2 per min at room temperature.

3.2.7 Statistical analysis

Data in the tables and text are presented as mean values \pm standard deviation. Enzyme activities and protein solubility were analysed using a model with the fixed effects of muscle, time of display, slaughter day, and all two-way interactions. The other variables were analysed using a model with muscle, slaughter day and muscle \times slaughter day as fixed effects. The effect of slaughter day was significant for most of the parameters but interaction terms with slaughter day were significant in only a few cases. The effects of slaughter day are not further discussed here and did not interfere with the effects of muscle and time of display. Post-hoc tests were performed at a significance level of $P < 0.05$ using the Tukey correction for multiple tests. Pearson correlations were used for testing the correlations between antioxidant enzyme activities and meat quality traits. The enzyme activity variables and most of the meat quality variables ($n=15$ in total) that were measured on the three muscles ($n=120$ observations in total), thus excluding pH and temperature measurements, were also submitted to principal component analysis (PCA) for further examining the interrelationships. In this analysis, if two variables from the same family of measurements, e.g. colour, were closely related ($r > |0.75|$), only one variable was included. The analyses were done using SAS® Enterprise Guide®, version 4.3 (SAS Institute Inc., Cary, NC, USA).

3.3 RESULTS AND DISCUSSION

3.3.1 Post-mortem pH and temperature fall and protein solubility

In Fig. 3.1 the pH versus temperature fall is presented. The pH at 1, 3 and 48 hours post-mortem was not significantly different between the IBF and the LD (6.61 ± 0.16 versus 6.47 ± 0.27 , 5.64 ± 0.22 versus 5.69 ± 0.23 and 5.51 ± 0.09 versus 5.56 ± 0.13 for pH1, pH3 and pH48 respectively, $P > 0.05$), whereas pH at 5 hours post-mortem was lower in the IBF than in the LD (5.42 ± 0.07 versus 5.55 ± 0.12 , $P < 0.001$). Temperature was not different at 1 hour post-mortem (39.9 ± 0.6), but was higher throughout the cooling period for IBF compared to LD (39.3 ± 1.0 versus 36.0 ± 1.4 , 36.7 ± 0.9 versus 30.1 ± 1.7 , and 4.6 ± 1.1 versus 1.3 ± 0.6 for 3, 5 and 48 hours post-mortem respectively; all $P < 0.001$). Due to the relatively slow temperature fall and fast pH fall, conditions for heat shortening were met for a certain time (Thompson, 2002). The duration of heat shortening was longer in IBF (3.88 hours) than in LD (1.20 hours; $P < 0.001$). Temperature and pH were not followed in the OBF, but it can be assumed that no heat shortening occurred in the exterior part of the BF muscle due to the faster cooling rate. The pH values in the LD at 1 and 3 hours post-mortem were lower than what we observed earlier in double-muscled bulls (Steen et al., 1997), and it is clear that the very low pH values at 3 and 5 hours post-mortem are characteristic of a (very) fast rate of glycolysis for beef. In the classification used by O'Halloran et al. (1997), our samples would also be rated as fast glycolysing.

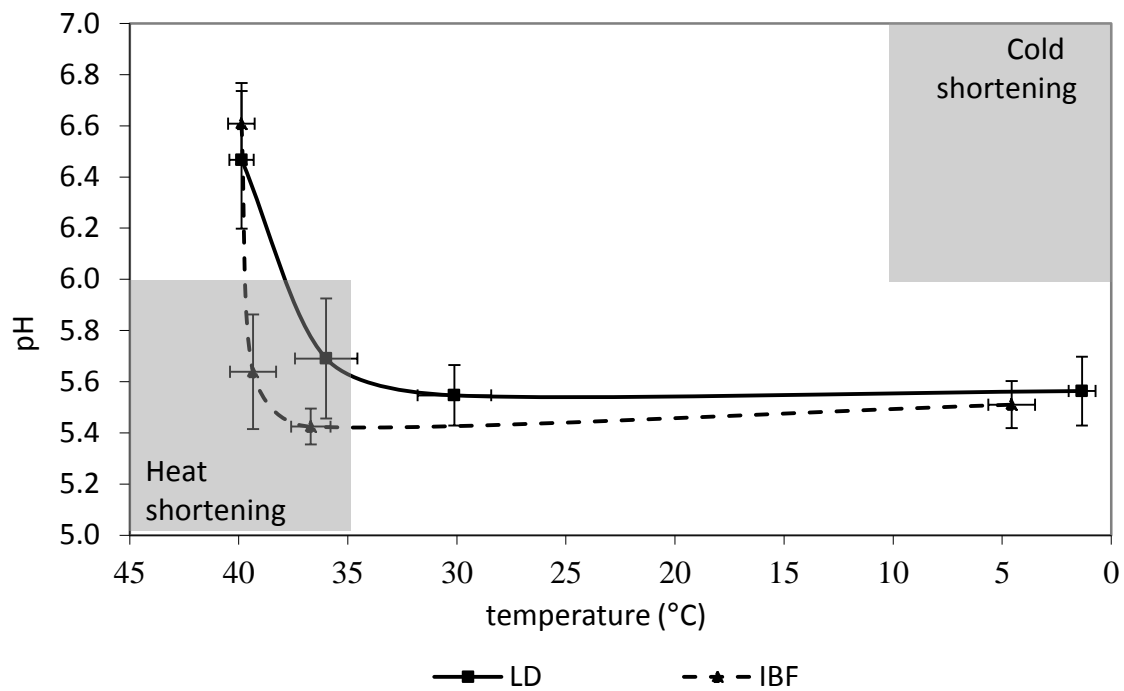


Fig. 3.1 Mean values ($n = 40$) for post-mortem pH and temperature for the inner *M. Biceps femoris* (IBF) and *M. Longissimus dorsi* (LD) muscle. Error bars represent standard deviations. The marks from left to right correspond to the measurements at 1, 3, 5 and 48 hours post-mortem. pH was different between the two muscles at 5 hours post-mortem ($P < 0.001$), whereas temperature was different at 3, 5 and 48 hours post-mortem ($P < 0.001$)

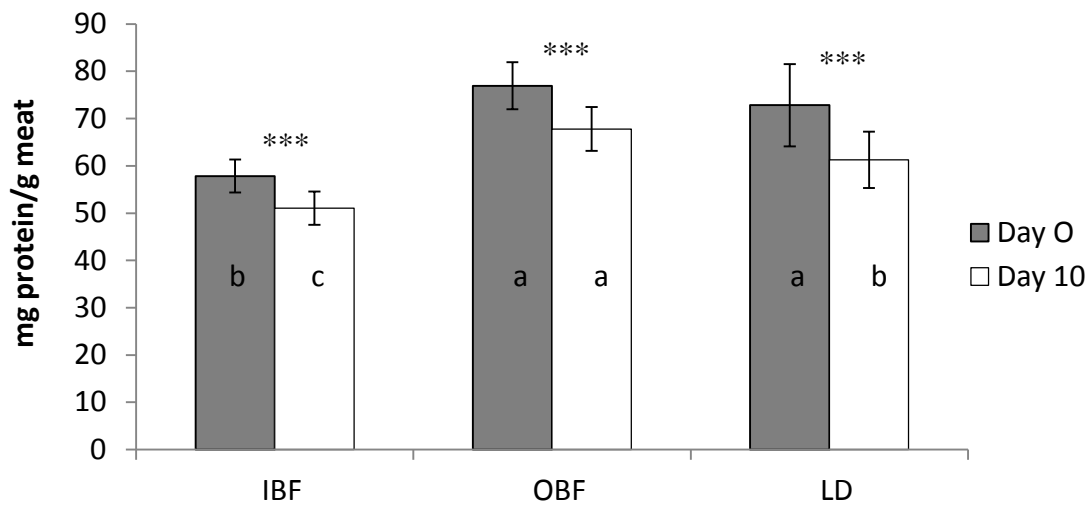


Fig. 3.2 Mean values (n = 40) for protein solubility (mg protein/g meat) according to muscle type (inner *M. biceps femoris*, IBF; outer *M. biceps femoris*, OBF; *M. Longissimus dorsi*, LD) and time of display. Error bars represent standard deviations. ^{a,b,c} Mean values within time of display with a different superscript are significantly different at $P < 0.001$. *** denotes significant effect of time of display within muscle ($P < 0.001$)

Protein solubility at day 0 of display was lower in the IBF compared to the OBF and LD, which were not different, whereas at day 10 of display the three muscles differed in the order $IBF < LD < OBF$ ($P < 0.001$) (Fig. 3.2), suggesting more protein denaturation in the IBF resulting from heat shortening. Moreover, protein solubility at day 10 of display was lower than at day 0 in all muscles ($P < 0.001$). It should be mentioned that protein solubility was measured in a low-ionic strength buffer so that mainly sarcoplasmic proteins and enzymes are covered by this method. A more intense protein denaturation resulting from a faster post-mortem pH decline was suggested to be the cause of reduced activity of several proteolytic and lipolytic enzymes in pork (Claeys et al., 2001).

We therefore hypothesised that increased protein denaturation, assessed by a reduced protein solubility in the present study, might affect the activity of endogenous antioxidant enzymes.

3.3.2 Colour, oxidative stability and α -tocopherol content

The average lightness (L^*) value of the IBF at day 0 of display was higher than for the LD and OBF and remained higher throughout the display period ($P < 0.001$), illustrating the paler colour in the deeper muscles and the resultant two-toning in the BF muscle (Table 3.1). This is in line with the expectation from the faster pH fall and slower temperature fall (Fischer & Hamm, 1980), and the concomitant heat shortening condition. Steen et al. (1997) also reported a significant negative correlation between the L^* value and pH values at different times post-mortem in LD of double-muscled bulls. At the start of display, the colour a^* value (redness) was lower for the IBF than for the OBF and LD, which did not differ ($P < 0.001$). IBF decreased more in red colour (Δa^* value) during the first 7 days of display and a higher formation of metmyoglobin was noticed compared to the other two muscles ($P < 0.001$). In line here with, the chroma values were lower for the IBF compared to the OBF and LD during display ($P < 0.001$). These findings illustrate the lower colour stability of the IBF. McKenna et al. (2005) compared the colour stability of 19 bovine muscles, and ranked the LD among the “high colour stability” muscles, and the BF among the “low colour stability” muscles. These authors attributed discolouration differences between muscles to the amount of reducing activity relative to the oxygen consumption rate. Muscles of high colour stability have in general higher reducing ability

and oxygen penetration depth, and lower oxygen consumption rate and myoglobin content than muscles of low colour stability. No measurements of reducing activity, oxygen consumption rate or oxygen penetration depth were performed in the present study; hence we cannot exclude that these factors did not interfere in our results. However, our findings comparing the outer and inner part of the BF with the LD indicate that within-muscle variation may be larger than differences between muscles, mediated by differences in post-mortem cooling rate. The precise sampling location in the BF was not specified in the study of McKenna et al. (2005), so within-muscle variation might also have affected their findings. Given the effect of post-mortem temperature decline on antioxidant enzyme activities (see further), one might speculate that enzymes involved in reductase activity are also affected by the unfavourable pH and temperature conditions in the inner part of the BF, contributing to the lower colour stability of this part of the muscle. Similar within-muscle variability in colour stability related to post-mortem cooling rate was reported by Sammel et al. (2002) for the inside and outside *M. Semimembranosus*.

TBARS values at day 0 of display were higher in the IBF and OBF compared with the LD muscle ($P < 0.001$; Table 3.1), while the value at day 10 of display was significantly higher in the IBF than the OBF and LD ($P < 0.001$). After 10 days of storage, TBARS values significantly increased in the three muscles ($P < 0.001$) as expected. In line with these results, Chen et al. (2010) reported that PSE pork (*M. Longissimus*) had higher TBARS values than normal pork, and Li and King (1996) related myosin denaturation with lipid oxidation in chicken thigh muscle that was incubated at 37 °C for up to 3 hours after storage at -85 °C. Also in the work of McKenna et al. (2005) on beef, muscles of lower

colour stability were characterised by higher oxidative rancidity. This relationship between lipid and myoglobin oxidation is well established for beef (Faustman et al., 2010).

The mean α -tocopherol content at day 0 of display was higher in the IBF than OBF and LD ($P<0.05$), whereas the latter two were not different (Table 3.1). It is well known that α -tocopherol is incorporated in muscle cell membranes and protects against lipid and pigment oxidation (Liu et al., 1995; Sales & Koukolová, 2011). The reason for the slightly higher α -tocopherol concentration in the inner part of the BF compared to the outer part is not clear, but illustrates that the lower oxidative stability observed in this part of the muscle is not related to a local difference in the concentration of antioxidants. This gives thus additional support to the detrimental effect of fast glycolysis on the oxidative stability of meat samples observed in this study.

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Table 3.1 Colour parameters, thiobarbituric acid reactive substances (TBARS) and α -tocopherol content during display in inner *M. Biceps femoris* (IBF), outer *M. Biceps femoris* (OBF) and *M. Longissimus dorsi* (LD) of young double-muscled Belgian Blue bulls (mean \pm standard deviation, n = 40)

	IBF	OBF	LD	P
L^* d0	49.9 \pm 3.0 ^a	37.4 \pm 2.5 ^c	39.2 \pm 4.0 ^b	<0.001
L^* d1	50.6 \pm 3.0 ^a	38.8 \pm 2.3 ^c	40.3 \pm 3.8 ^b	<0.001
L^* d7	52.4 \pm 3.0 ^a	40.0 \pm 2.1 ^b	41.2 \pm 3.6 ^b	<0.001
L^* d10	52.8 \pm 3.5 ^a	40.1 \pm 2.3 ^b	41.4 \pm 4.1 ^b	<0.001
a^* d0	20.4 \pm 2.5 ^b	21.3 \pm 1.8 ^{ab}	21.8 \pm 1.7 ^a	0.004
a^* d1	19.1 \pm 2.4 ^b	22.9 \pm 1.8 ^a	22.4 \pm 1.9 ^a	<0.001
a^* d7	10.0 \pm 1.8 ^c	19.6 \pm 2.3 ^a	17.8 \pm 3.1 ^b	<0.001
a^* d10	7.1 \pm 1.4 ^c	13.4 \pm 4.4 ^a	11.1 \pm 4.5 ^b	<0.001
Δa^* d1–d7 (/d)	-1.32 \pm 0.27 ^c	-0.56 \pm 0.41 ^a	-0.76 \pm 0.36 ^b	<0.001
Chroma d0	30.6 \pm 2.7 ^a	29.5 \pm 2.5 ^b	30.3 \pm 2.0 ^{ab}	0.021
Chroma d1	29.1 \pm 2.6 ^b	31.1 \pm 2.1 ^a	30.7 \pm 1.8 ^a	<0.001
Chroma d7	20.9 \pm 1.8 ^c	27.7 \pm 2.5 ^a	26.0 \pm 2.9 ^b	<0.001
Chroma d10	19.7 \pm 1.5 ^b	22.1 \pm 4.1 ^a	20.2 \pm 4.0 ^b	<0.001
%MetMb d0	22.9 \pm 3.6 ^a	15.3 \pm 2.2 ^b	16.1 \pm 3.4 ^b	<0.001
%MetMb d1	24.4 \pm 2.7 ^a	17.6 \pm 2.3 ^b	18.5 \pm 3.3 ^b	<0.001
%MetMb d7	44.8 \pm 6.5 ^a	24.2 \pm 3.6 ^c	27.3 \pm 6.9 ^b	<0.001
%MetMb d10	60.7 \pm 8.7 ^a	40.9 \pm 14.9 ^c	48.4 \pm 15.3 ^b	<0.001
Δ %MetMb d1–d7 (/d)	3.26 \pm 1.03 ^a	0.92 \pm 0.42 ^b	1.22 \pm 0.80 ^b	<0.001
TBARS d0 (μ g/g)	0.35 \pm 0.12 ^a	0.32 \pm 0.09 ^a	0.23 \pm 0.08 ^b	<0.001
TBARS d10 (μ g/g)	3.29 \pm 1.45 ^a	2.05 \pm 1.11 ^b	0.99 \pm 0.83 ^c	<0.001
α -tocopherol d0 (μ g/g)	2.77 \pm 0.79 ^a	2.35 \pm 0.66 ^b	2.28 \pm 0.74 ^b	0.017

^{a, b, c} Within a row, mean values with different superscripts differ significantly at $P < 0.05$

3.3.3 Antioxidant enzyme activities

At day 0 of display, the LD and IBF had a higher SOD activity than the OBF ($P<0.001$) (Table 3.2), whilst pH and temperature were higher and lower respectively in the latter muscle compared to the other muscles. The present results thus indicate that SOD activity was not affected by the variation in glycolysis between muscles. This is in agreement with Rotilio et al. (1972) who reported that the SOD activity from human and yeast are invariant with pH over a wide range (5–10), and Lyons et al. (1996) who found that the SOD activity from bovine erythrocytes was the same at pH 7.0 and pH 5.5. Nevertheless, after 10 days of display, the SOD activity was lower in the three muscles when compared with day 0 of display ($P<0.001$). Also in the study of Renerre et al. (1996), SOD activity in different beef muscles decreased significantly between day 1 and day 8 of cold storage.

The IBF had a lower activity of GSH-Px than the OBF and LD muscles at day 0 and 10 of display ($P<0.05$) (Table 3.2). This suggests that the higher temperature and lower pH in the IBF had a negative effect on GSH-Px activity. This result is in accordance with Naash and Anderson (1984) who reported that GSH-Px activity from frog retina homogenates increased steadily in buffer conditions from around pH 5.5 up to pH 8.0; moreover, there was a rapid increase above pH 7.0 in activity with increasing buffer pH. Contrary to the results obtained with SOD activities, there was an overall significant increase in GSH-Px activity at day 10 compared to day 0 of display ($P<0.001$); however the post-hoc comparison of means test did not reveal significant differences for the muscles separately. In their study, Renerre et al. (1996) observed that the GSH-Px activity

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remained stable during cold storage of different beef muscles for 8 days. It can thus be noted that GSH-Px activity seems to be rather stable in beef during cooled storage.

At day 0 of display, the order of CAT activity in the three muscles was similar to the data obtained for GSH-Px activity, i.e. the IBF muscle had a lower activity of CAT than the OBF and LD ($P < 0.001$). CAT activity thus seems also to be affected by the glycolysis conditions. However, contrary to GSH-Px, the CAT activity decreased in the three muscles during 10 days of display ($P < 0.001$). In contrast, Renerre et al. (1996) found that after 8 days of cold storage, the CAT activities were similar to those obtained at day 1 post-mortem.

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Table 3.2 Activities (Unit¹/g) of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in inner *M. Biceps femoris* (IBF), outer *M. Biceps femoris* (OBF) and *M. Longissimus dorsi* (LD) at day 0 (48 h pm) and day 10 of display in young double-muscled Belgian Blue bulls (mean \pm standard deviation, n = 40)

Enzyme	Display	IBF	OBF	LD
GSH-Px	d0	1.33 \pm 0.27 ^b	1.51 \pm 0.30 ^a	1.57 \pm 0.33 ^a
	d10	1.50 \pm 0.34 ^b	1.70 \pm 0.28 ^a	1.70 \pm 0.32 ^a
	$P_{\text{muscle}} < 0.001$; $P_{\text{time}} < 0.001$; $P_{\text{muscle} \times \text{time}} = 0.766$			
CAT	d0	99.0 \pm 24.3 ^{b,x}	132.2 \pm 32.1 ^{a,x}	123.9 \pm 26.9 ^{a,x}
	d10	75.8 \pm 22.6 ^{ab,y}	85.7 \pm 27.6 ^{a,y}	69.3 \pm 17.5 ^{b,y}
	$P_{\text{muscle}} < 0.001$; $P_{\text{time}} < 0.001$; $P_{\text{muscle} \times \text{time}} < 0.001$			
SOD	d0	65.5 \pm 9.7 ^{a,x}	57.6 \pm 7.6 ^{b,x}	68.5 \pm 10.8 ^{a,x}
	d10	44.4 \pm 7.9 ^{b,y}	43.0 \pm 6.9 ^{b,y}	50.7 \pm 9.1 ^{a,y}
	$P_{\text{muscle}} < 0.001$; $P_{\text{time}} < 0.001$; $P_{\text{muscle} \times \text{time}} = 0.042$			

^{a, b, c} Within a row, mean values with different superscripts differ significantly at $P < 0.05$.

^{x,y} Within muscle and enzyme, mean values with different superscripts differ significantly at $P < 0.05$. ¹ One unit of SOD was defined as the amount of extract needed to inhibit the rate of oxidation by the control (no SOD) by 50%. One unit of GSH-Px activity was defined as the amount of extract needed to oxidise 1 μmol of NADPH per min at 25 °C. One unit of CAT was defined as the amount of extract needed to decompose 1 μmol of H_2O_2 per min at room temperature

3.3.4 Relationship between antioxidant enzyme activities and meat quality traits

The differences between muscles in post-mortem pH and temperature fall discussed above revealed a possible dependence of the activity of GSH-Px and CAT on the early post-mortem conditions. Meat colour and lipid stability was also negatively affected by heat shortening conditions. Consequently, if the antioxidant enzymes analysed in the present study play a role in maintaining meat oxidative stability, one can expect relationships between the antioxidant enzyme activities and meat quality traits in a set of carcasses. Pearson correlation coefficients were calculated for this purpose. The correlation coefficients were calculated across muscles; hence the co-variances do not only reflect variation between carcasses but also partly result from the differences between muscles.

For the three enzymes, there was a significant positive relationship between the activity at day 0 versus day 10 of display ($r=0.72$, 0.46 and 0.26 for GSH-Px, CAT and SOD respectively; $n=120$; $P<0.01$). Correlation coefficients between the activities of different enzymes at the same or a different time point were all lower than $|0.2|$ and not meaningful. The enzyme activities were poorly related to pH values, but showed on the other hand an overall negative relationship with temperature post-mortem and with the duration of heat shortening, except for CAT at day 10 that was not or weakly positively related to these variables. The highest correlations with temperature values were observed for CAT at day 0, GSH-Px at day 0 and SOD at day 10 ($r=-0.37$, -0.28 and -0.28 with temperature 5 hours post-mortem respectively; $r=-0.54$, -0.27 and -0.38 with

temperature 48 hours post-mortem respectively; $n=80$; $P<0.05$). Correlation coefficients with the duration of heat shortening were of similar magnitude.

The relationships between enzyme activities and meat quality traits across the three muscles were most obvious and of the same sign for GSH-Px at day 0 and 10 and CAT at day 0, e.g. these enzyme activity values were negatively related to the colour L^* value, %MetMb and TBARS formation, and were positively related to protein solubility values. These correlation coefficients were in the range $r=|0.2|$ to $|0.4|$ ($P<0.01$). On the other hand, SOD activity at day 0 was positively related to metmyoglobin formation and negatively to protein solubility, whereas SOD at day 10 was related to TBARS formation ($r=|0.2|$ to $|0.3|$; $P<0.01$). Among meat quality traits, the highest correlation coefficients (all $P<0.01$) were noticed for protein solubility at day 0 with L^* value ($r=-0.84$), metmyoglobin formation ($r=-0.71$) and TBARS at day 10 ($r=-0.47$). The L^* value was also strongly positively related to metmyoglobin formation ($r=0.71$) and TBARS at day 10 ($r=0.48$). TBARS at day 10 were related to both %MetMb at day 0 and metmyoglobin formation during display ($r=0.51$ and $r=0.49$ respectively). These relationships among meat quality traits are thus stronger than with the enzyme activities, suggesting that variation in the antioxidant enzyme activities might contribute to variation in meat oxidative stability, but is for sure not the only responsible factor. In the present study, the α -tocopherol content was not related meaningfully to TBARS and metmyoglobin formation, which is surprising in view of the well-known and important role of vitamin E in protection against colour and lipid oxidation (Liu et al., 1995; Sales & Koukolová, 2011). However, it should be mentioned that animals in the present study originated from commercial farms and there was no control on the feeding regimes and

the dietary levels of vitamin E. The above findings are illustrated in Fig. 3.3, in which the variable scores are presented for principal component (PC) 1 and 2 of the PCA. PC1 and PC2 explained 30.8% and 12.2% of the total variance, whereas PC3 and PC4 explained 10.6% and 10.0% respectively. PC1 is characterised by high scores for protein solubility, colour L^* value and %MetMb, and somewhat lower scores for TBARS. PC1 is thus reflecting the oxidative stability. PC2 was mainly associated with SOD and GSH-Px, α -tocopherol and to a lesser extent TBARS. The enzyme activity variables were not associated with one PC, e.g. SOD and GSH-Px were associated with PC2, PC4 and to a lesser extent PC1. CAT was associated with PC3, that was further mainly characterised by the colour a^* value, and with PC1 for the initial activity. A fifth PC was mainly associated with α -tocopherol. Taken together, these findings illustrate that variation in the endogenous antioxidant enzyme activities is only partly related to meat oxidative stability, and the precise contribution of these enzymes in retarding oxidative damage during storage of meat post-mortem needs further research.

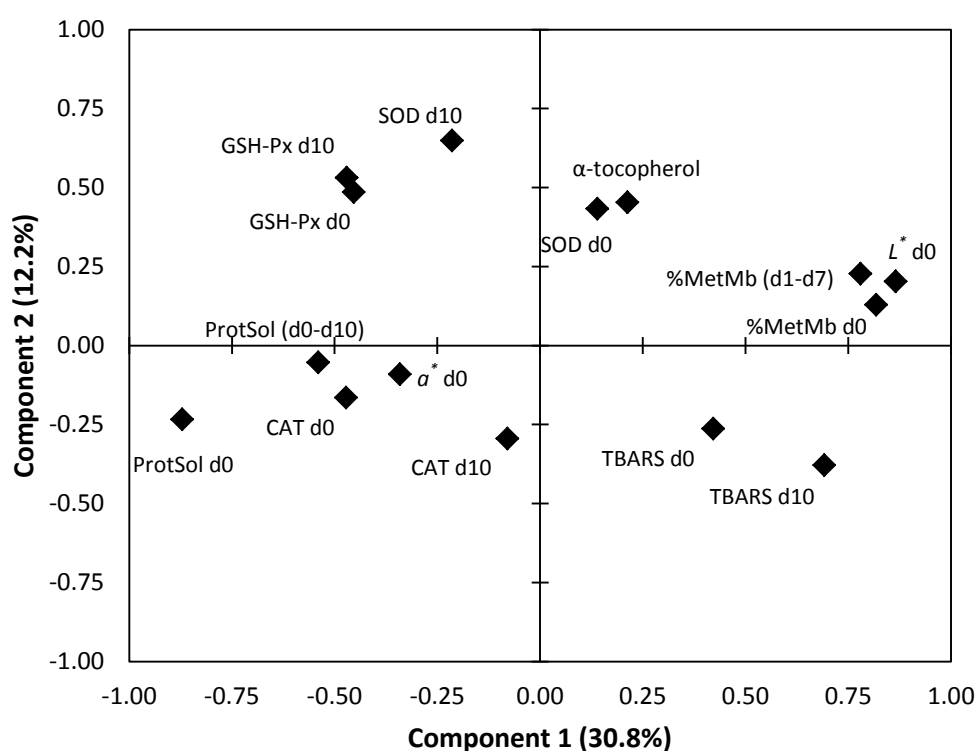


Fig. 3.3 Variable scores for Principal Component 1 and 2 of the Principal Component Analysis. Legend: ProtSol, protein solubility; d, day of display; %MetMb, % metmyoglobin

3.4 CONCLUSION

The activities of CAT and GSH-Px were lower, whereas lipid oxidation and formation of metmyoglobin were higher in the inner part of the *M. Biceps femoris* compared to the outer part of the *M. Biceps femoris* and the *M. Longissimus*, associated with an increased degree of heat shortening and protein denaturation. The lower CAT and GSH-Px activities in the inner part of the *M. Biceps femoris* might result from increased denaturation induced during the longer heat shortening time. However, heat shortening did not affect the SOD activity and the α-tocopherol content. The relationships between

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the enzyme activities and meat quality traits observed in this study suggest that these endogenous antioxidant enzymes are implicated in the protection against oxidative damage during storage of meat; however, the relative importance of this contribution needs to be further examined.

**EFFECT OF HIGH POST-MORTEM TEMPERATURE ON ANTIOXIDANT
ENZYME ACTIVITIES AND MEAT QUALITY IN PORK**

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EFFECT OF HIGH POST-MORTEM TEMPERATURE ON ANTIOXIDANT ENZYME ACTIVITIES AND MEAT QUALITY IN PORK

ABSTRACT

The objective of this study was to investigate the influence of high post-mortem (pm) temperature on the activity of antioxidant enzymes and meat quality in pork *M. Longissimus dorsi* (LD). Pork LD was sampled from the left and right side of 15 pig carcasses at 30 min pm. The LD of one carcass side was kept at 40 °C in an hot air oven for 4 hours and then moved to a chiller at 4 °C until 24 hours pm (delayed chilling), whereas the other side was immediately placed in the same chiller (normal chilling). Delayed chilling resulted in PSE characteristics, heat shortening, lower protein solubility and lower concentration of individual proteins in both of sarcoplasmic and myofibrillar fractions compared to normal chilling ($P<0.05$). The activities of catalase, superoxide dismutase and glutathione peroxidase at 24 hours after cutting were lower in the delayed chilling compared to the normal chilling treatment ($P<0.05$). Colour L^* values were higher during 8 days of display ($P<0.001$) and a^* values were significantly lower from day 4 of display on ($P<0.05$) in the delayed compared to the normal chilling treatment. TBARS values were lower after 8 days of display in the delayed chilling treatment ($P<0.01$). It was concluded that induction of PSE characteristics by high pm temperature reduced the activity of antioxidant enzymes, protein solubility and colour stability, but lipid and protein oxidation were not affected.

4.1 INTRODUCTION

The combination of high temperature and fast pH decline in muscle early post-mortem (pm) leads to increased protein denaturation compared to when a normal onset of rigor mortis is present. This results in the occurrence of PSE meat, i.e. pale, soft and exudative meat (Bendall & Swatland, 1988). Several animal factors and pre-slaughter conditions contribute to the development of PSE (De Smet et al., 1996; Schaefer et al., 2001). Carcass cooling is one of these factors that needs to be optimised in order to guarantee meat quality. Fast cooling of carcasses does not allow to reverse but it may alleviate to some extent the effects of adverse pre-slaughter conditions on meat quality (Offer, 1991; Van Moeseke et al., 2001; Xu et al., 2012). For research purposes, installing different chilling regimes allows to induce different meat qualities, e.g. hot boning followed by rapid chilling may result in improved meat quality, whereas delayed chilling is expected to worsen meat quality.

The causes and characteristics of PSE meat have been abundantly studied but the concomitant changes in oxidative stability upon further storage or display have been less well documented. Meat oxidative deterioration leads to a loss of nutritional value and reduced sensory quality. Several mechanisms are present in muscle to protect against oxidation including the endogenous antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). In a previous study in beef, we found that the activities of CAT and GSH-Px were lower, whereas lipid oxidation and formation of metmyoglobin were higher in the deeper laying part of the *M. Biceps femoris* compared to the superficial part, which was associated with an increased degree of heat shortening and protein denaturation (Pastsart et al., 2013) as indicated in

Chapter 3. Protein denaturation resulting from severe heat shortening may affect the activity of these endogenous antioxidant enzymes. Hence, it may be argued that heat shortening conditions result in a reduced activity of these enzymes and thereby negatively affect the oxidative stability of meat.

Therefore, the objective of this study was to investigate the influence of two chilling treatments, i.e. high temperature early pm (40 °C for 4 hours and then 4 °C until 24 hours) versus a normal chilling regime (4 °C for 24 hours), on antioxidant enzyme activities and meat quality in pork *M. Longissimus dorsi* (LD) with emphasis on the meat oxidative stability.

4.2 MATERIALS AND METHODS

4.2.1 Animals and meat samples

A total of 15 pigs originating from commercial farms in Belgium were slaughtered according to common practices on two days in the same slaughterhouse. A piece of 30 cm long of the LD was cut from the left and right side of the carcasses within 30 min pm and kept in a plastic bag. The LD muscle of one carcass side was kept in an hot air oven at 40 °C for 4 hours and then moved to the chilling room at 4 °C until 24 hours pm (delayed chilling). The other carcass side was immediately placed in the chilling room at 4 °C for 24 hours (normal chilling). The samples for analysis of enzyme activity were taken at 0, 4 and 24 hours after cutting the LD (30 min pm) and put in liquid nitrogen, then stored at -80 °C until analysis. For the simulated retail display, meat samples were

wrapped in oxygen permeable foil and displayed at 4 °C under constant fluorescent light (approximately 1200 lux) for 8 days.

4.2.2 Meat quality measurements

The temperature and pH were measured at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 hours after cutting the LD (30 min pm). The duration of 'heat shortening' was calculated based on the definition of pH < 6.0 and temperature > 35 °C (Thompson, 2002). The Pork Quality Meter (PQM) was used to measure the conductivity at 24 hours after cutting. Meat colour was measured with a Hunterlab Miniscan colour meter (D65 light source, 10° standard observer, 45°/0° geometry, 1-inch light surface, white standard) at 0, 1, 2, 3, 4, 5, 6, 7 and 8 days of display. The percentage of metmyoglobin (%MetMb) was examined at 0, 1, 2, 3, 4, 5, 6, 7 and 8 days of display using reflectance values at the wavelengths 520, 530, 570, 580 and 700 nm using the formulas as modified by Lindahl et al. (2001). Determination of drip loss, thawing loss, and cooking loss was carried out at 24 hours after cutting as described by Uytterhaegen et al. (1994).

4.2.3 Lipid oxidation

Lipid oxidation was assessed spectrophotometrically and expressed as µg malondialdehyde (MDA) / g meat on the samples of day 8 of display by the thiobarbituric acid reactive substances (TBARS) method as described by Tarladgis et al. (1960).

4.2.4 Protein oxidation

Protein oxidation was evaluated by determining the carbonyl content of samples of day 0 and day 8 of display according to Ganhão et al. (2010). The measurement of protein carbonyls was done following their covalent reaction with 2,4-dinitrophenylhydrazine (DNPH). This reaction leads to the formation of a stable 2,4-dinitrophenylhydrazone product. Total carbonyl content was quantified spectrophotometrically at 370 nm, using a molar absorption coefficient of 21.0/(mM.cm) and expressed as nmol DNPH incorporated/mg protein by using the formula of Jongberg et al. (2011).

$$\frac{C_{hydrazone}}{C_{protein}} = \frac{A_{370}}{\epsilon_{hydrazone,370} \cdot (A_{280} - A_{370} \cdot 0.43)} \cdot 10^6$$

Where, $\epsilon_{hydrazone,370}$ is $21000 \text{ M}^{-1}\text{cm}^{-1}$, and $0.43 = \epsilon_{hydrazone,280} / \epsilon_{hydrazone,370}$.

4.2.5 Protein solubility and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sarcoplasmic and myofibrillar protein solubility was measured on 24 hours pm samples according to Claeys et al. (2002) in a low- and high-ionic strength solution respectively, and was expressed as mg protein/g meat. More protein denaturation is assumed to result in lower protein solubility. Briefly, 3 g of minced meat sample was homogenized in 30 ml of cold (3 °C) solution (150 mM NaCl and 0.01 mM iodo-acetic acid), followed by centrifugation at 2000g for 10 min. The supernatant was filtered and considered as the soluble sarcoplasmic protein fraction. The pellet was re-suspended in another 30 ml of the same solution, centrifuged and the supernatant was decanted. The pellet was re-

suspended by using ultra-turrax at the lowest speed (11000 rpm) in 45 ml of a buffer solution at pH 5.5 (prepared with 0.1 M citric acid, 1 mM EDTA, 0.4 M NaCl and 0.01 mM iodo-acetic acid). The tubes were placed in a water bath at 20 °C for 2 hours, mixed manually every 30 min and centrifuged at 5000g for 20 min. The supernatant was carefully decanted and considered as the soluble myofibrillar protein fraction. The protein content of both solutions of sarcoplasmic and myofibrillar proteins were determined using the biuret method (Herbert et al., 1971); the sum being the total protein solubility. Both protein fractions were subsequently separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Claeys et al. (1995). The identification of the protein bands was done by comparison with protein patterns reported in previous studies (Savage et al., 1990; Claeys et al., 1995). The concentrations of the separated proteins were calculated based on the peak area of the protein bands relative to the total peak area and the total amount of soluble protein in the meat sample.

4.2.6 Antioxidant enzyme activity assays

Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) activities were determined on samples at 0, 4 and 24 hours after cutting the LD (30 min pm). After thawing, the muscle samples were kept on ice during the procedure. A 5 g muscle sample was homogenized in 10 ml of 0.05 M phosphate buffer (pH 7.0) and centrifuged at 4 °C for 20 min at 7000g. The supernatant fraction was filtered through glass wool before determining enzyme activities.

The SOD activity assay was performed as described by Marklund and Marklund (1974) by measuring the inhibition of pyrogallol autoxidation. A unit of enzyme activity was defined as the amount of sample needed to inhibit the reaction by 50%. The activity of GSH-Px was determined by measuring the oxidation of NADPH. One unit of GSH-Px activity was defined as the amount of extract required to oxidize 1 μmol of NADPH per min at 25 °C (Hernandez et al., 2004). The CAT activity was determined according to the method of Aebi (1983). One unit of CAT activity was defined as the amount of sample required to decompose 1 μmol of H_2O_2 per min at room temperature.

4.2.7 Statistical analysis

Enzyme activities were analyzed using a model with the fixed effects of chilling treatment, sampling time, slaughter day, and their interaction terms. Meat quality variables were analyzed with a model including the fixed effects of chilling treatment, slaughter day and their interaction. For a^* and L^* values during display this model was extended with the repeated measures effect of day of display. The General Linear Model (GLM) procedure of SAS® Enterprise Guide®, version 4.3 (SAS Institute Inc., Cary, NC, USA) was used. Post hoc comparison of means tests were performed at a significance level of $P < 0.05$ using Tukey.

4.3 RESULTS AND DISCUSSION

4.3.1 Post-mortem pH and temperature fall and meat quality traits

In Figure 4.1 the pH versus temperature fall in pork LD is presented. The duration of heat shortening was estimated at 3.1 ± 0.67 hours in the delayed chilling treatment, whereas no heat shortening was observed in the normal chilling treatment.

The mean values for meat quality traits according to the chilling treatments are shown in Table 4.1. PQM values were higher in the delayed chilling compared to the normal chilling treatment whereas sarcoplasmic and myofibrillar protein solubility was lower ($P < 0.001$). Meat in the delayed chilling treatment was much paler with a difference of 7 units in the L^* value at day 1 of display ($P < 0.001$), whereas there was no difference for the initial a^* value (Fig. 4.2A). On the other hand, ultimate pH, drip loss, cooking loss and thawing loss were not significantly different between the two chilling treatments. The lower protein solubility and higher PQM values indicate that the high pm temperature increased protein denaturation and cell membrane disruption respectively, which was accompanied by only a numerical increase in drip loss. However, it should be mentioned that drip losses assessed in the present study were not representative for the total fluid losses that occurred during storage. The experimental procedure involved cutting the loins early pm and storing them in plastic bags during the chilling treatments. It was visually observed that considerably more fluid losses accumulated in the bags of the delayed chilling treatment compared to the normal chilling treatment. Unfortunately these fluid losses were not recorded. The subsample for drip loss determination was subsequently taken at 1 day pm after the chilling treatment. The preceding fluid losses during chilling have probably caused an underestimation of the subsequent drip loss.

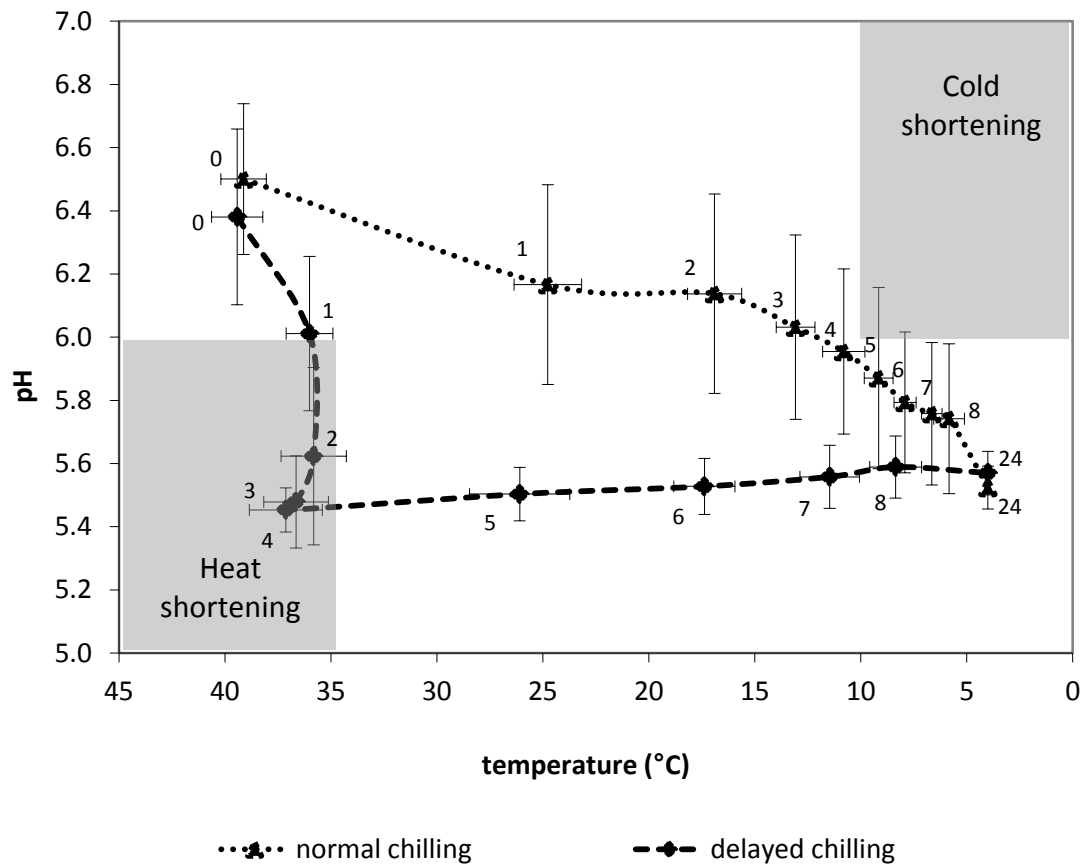


Fig. 4.1 Mean values ($n = 15$) for pH and temperature in pork LD according to chilling treatment. Error bars represent standard deviations. The marks from left to right correspond to the measurements at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 hours after cutting the LD (30 min pm)

Table 4.1 Effect of delayed versus normal chilling on meat quality traits in pork LD (mean \pm SD, n=15)

	Chilling regime		P-value
	Delayed	Normal	
PQM 24 h pm (μ S)	16.07 \pm 1.30	6.63 \pm 2.88	<.001
pH 24 h pm	5.57 \pm 0.07	5.53 \pm 0.06	0.116
Drip loss (%)	5.68 \pm 1.04	5.13 \pm 1.29	0.188
Thawing loss (%)	8.88 \pm 1.45	8.83 \pm 2.13	0.932
Cooking loss (%)	25.10 \pm 1.01	25.48 \pm 1.94	0.493
Sarcoplasmic protein solubility (mg protein/g)	62.28 \pm 4.07	74.11 \pm 2.43	<.001
Myofibrillar protein solubility (mg protein/g)	11.90 \pm 0.89	14.93 \pm 0.53	<.001
<i>L</i> * d0	59.47 \pm 3.52	51.21 \pm 2.95	<.001
TBARS d8 (μ g/g)	0.16 \pm 0.06	0.24 \pm 0.06	0.002
Protein carbonyls d0 (nmol/mg protein)	3.33 \pm 0.54	3.11 \pm 1.03	0.409
Protein carbonyls d8 (nmol/mg protein)	5.22 \pm 1.10	4.88 \pm 1.08	0.386

4.3.2 Antioxidant enzyme activities

Table 4.2 indicates that the enzyme activities were all influenced by chilling treatment ($P < 0.02$), sampling time ($P < 0.001$; except for GSH-Px, $P > 0.05$) and chilling treatment \times time ($P < 0.02$). For CAT and SOD, the activity measured at 24 hours after cutting in the delayed chilling treatment was significantly lower compared to the activity at earlier time points in this treatment and compared to the activities at all sampling times in the normal chilling treatment. For GSH-Px, the activity at 24 hours after cutting in the delayed chilling treatment was significantly lower than the activity at 4 and 24 hours

after cutting in the normal chilling treatment. This illustrates that the higher temperature early pm and the heat shortening conditions in the delayed chilling treatment reduced the activity of CAT, SOD and GSH-Px. This is in accordance with our previous study in beef (Pastsart et al., 2013), in which we found lower CAT and GSH-Px activities in the deeper laying part of the *M. Biceps femoris* compared to the superficial part, associated with a slower temperature fall and increased protein denaturation in the inner muscle part.

Table 4.2 Effect of delayed versus normal chilling on activities (Unit¹/g) of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in pig LD at 0, 4 and 24 hours after cutting the LD (30 min pm) (mean \pm SD, n = 15)

Chilling	Delayed			Normal			P-values		
Time	0h	4h	24h	0h	4h	24h	Chill	Time	CxT
CAT	2355 \pm 208 ^a	2347 \pm 241 ^a	1865 \pm 315 ^b	2336 \pm 210 ^a	2295 \pm 263 ^a	2289 \pm 209 ^a	0.022	<.001	<.001
SOD	21.7 \pm 5.4 ^b	23.8 \pm 6.1 ^{ab}	13.3 \pm 10.7 ^c	20.5 \pm 2.7 ^b	25.9 \pm 3.1 ^a	23.6 \pm 3.4 ^{ab}	<.001	<.001	<.001
GSH-Px	0.57 \pm 0.26 ^{ab}	0.53 \pm 0.20 ^{ab}	0.38 \pm 0.14 ^b	0.52 \pm 0.18 ^{ab}	0.60 \pm 0.20 ^a	0.65 \pm 0.20 ^a	0.018	0.611	0.016

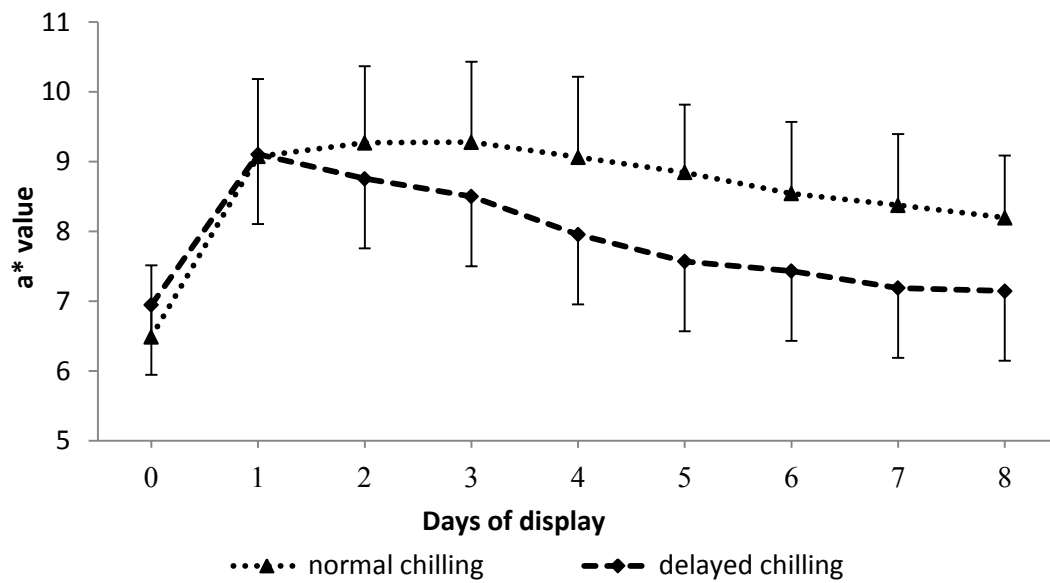
^{a, b, c} Within a row, mean values with different superscripts differ significantly at $P < 0.05$. ¹One unit of SOD was defined as the amount of extract needed to inhibit the rate of oxidation by the control (no SOD) by 50%. One unit of GSH-Px activity was defined as the amount of extract needed to oxidise 1 μ mol of NADPH per min at 25 °C. One unit of CAT was defined as the amount of extract needed to decompose 1 μ mol of H₂O₂ per min at room temperature

4.3.3 Lipid oxidation and discolouration

Figure 4.2 illustrates the effect of chilling treatment on the decrease in a^* value and the increase in metmyoglobin (%MetMb) during 8 days of display as a measure of colour stability. From day 4 of display on, the a^* values were lower in the delayed chilling compared to the normal chilling treatment ($P < 0.05$), in line with a higher formation of Metmyoglobin (%MetMb) at all days of display ($P < 0.01$). These results illustrate the faster pigment oxidation and lower colour stability in the delayed chilling treatment. In our previous study, we found that %Metmb was higher in the paler part of beef M. *Biceps femoris* muscle with a lower activity of antioxidant enzymes (Pastsart et al., 2013). Zhu and Brewer (1998) reported that the activity of metmyoglobin reductase was lower in PSE pork than in normal and DFD pork during 7 days of cold storage. This meat quality effect on metmyoglobin reductase may be due to enzyme (protein) denaturation early in the post-mortem period, which occurs to a greater degree in PSE carcasses where pH is declining rapidly while the carcass is still hot.

Surprisingly, the faster discolouration was not associated with higher lipid oxidation. On the contrary, TBARS values after 8 days of display were lower in the delayed chilling treatment ($P < 0.002$; Table 4.1). In a previous study in beef (Pastsart et al., 2013), we had indications that higher lipid oxidation was associated with reduced activity of antioxidant enzymes. In the present study, this did not appear to be the case, but on the other hand reduced colour stability and antioxidant enzymes activity were apparently correlated when comparing the chilling treatments.

(A)



(B)

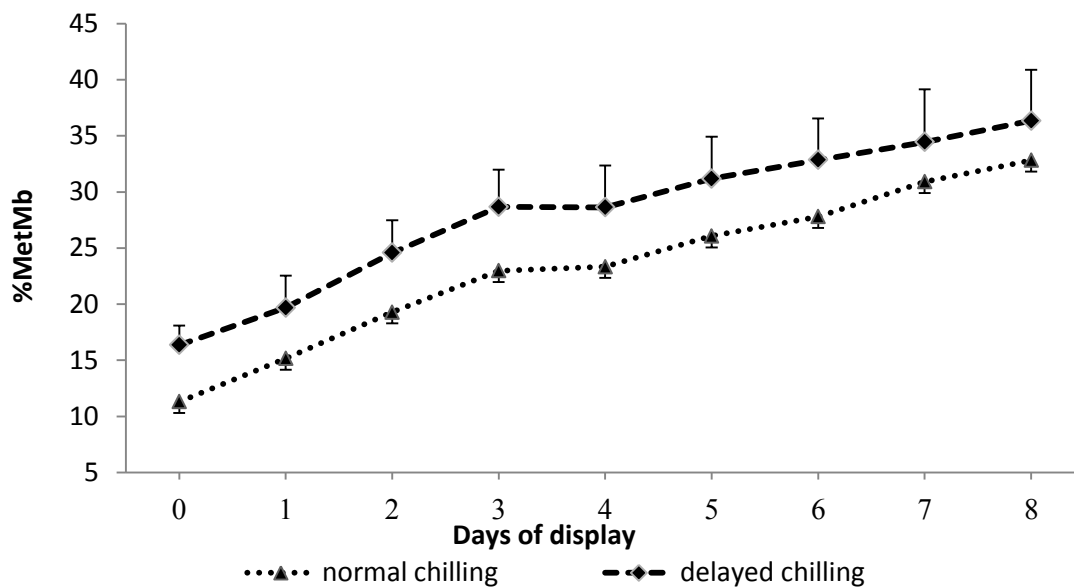


Fig. 4.2 Effect of delayed versus normal chilling on the colour a^* value (A) and %MetMb (B) in pork LD during display. The difference between normal and delayed chilling is significant from day 4 on for the a^* value and for all days for %MetMb ($P < 0.01$). Error bars represent standard deviations

4.3.4 Protein denaturation

As mentioned above, the solubility of the sarcoplasmic and myofibrillar protein fraction was significantly lower for the delayed chilling treatment compared to the normal chilling treatment ($P < 0.001$). This is in line with Claeys et al. (2002), who found a lower protein solubility and a lower concentration of several individual proteins in *Longissimus lumborum* of stress sensitive pigs compared to stress resistant pigs. It is well known that meat from stress-sensitive pigs is more prone to develop PSE meat characterized by a lower pH at 40 min pm, higher PQM values and higher colour L^* values.

Under the conditions of the present assay, the soluble sarcoplasmic protein fraction was approximately 5-fold larger than the soluble myofibrillar protein fraction. Myofibrillar and sarcoplasmic proteins denature to different degrees in muscle after death. In PSE meat, the denaturation occurs in both the myofibrillar and sarcoplasmic proteins, whereas in normal and DFD meat little or no denaturation of the myofibrillar proteins but some denaturation of sarcoplasmic proteins occurs (Lopez-Bote et al., 1989). Pre-rigor conditions in PSE muscle cause precipitation of the sarcoplasmic proteins onto the myofibrillar proteins inducing colour differences (Joo et al., 1999). Sarcoplasmic proteins appear thus to be more sensitive than myofibrillar proteins to conditions of fast post-mortem glycolysis.

We were interested in possible differences among individual proteins in solubility dependent on the chilling treatment. The identification of the different protein bands was done by comparison with patterns found in previous studies (Savage et al., 1990; Claeys et al., 1995), leaving some unknown bands. Figure 4.3 shows an example of the separation pattern for the two protein fractions. Figure 4.4 shows the concentration for

the individual proteins obtained from the SDS-PAGE patterns. The concentration of all proteins in the sarcoplasmic fraction, except C-protein, phosphoglucomutase, pyruvate kinase and myoglobin, was higher for the normal chilling than for the delayed chilling treatment ($P<0.05$). For the proteins in the soluble myofibrillar fraction, it was found that C-protein, α -actinin, phosphorylase b/ phosphorylase b kinase, actin, creatine phosphokinase/ phosphoglycerate kinase, β -tropomyosin and unknown protein (band 5) were higher in the normal chilling meat than in the delayed chilling meat ($P<0.01$); only lactate dehydrogenase was lower in the normal chilling meat ($P<0.01$). This suggests a high degree of denaturation of lactate dehydrogenase which is a sarcoplasmic protein, and which, after precipitation, can be found back in the myofibrillar fraction. There were some more sarcoplasmic proteins that precipitated onto the myofibrillar fraction, for example phosphorylase b/ phosphorylase b kinase, pyruvate kinase, phosphoglucose isomerase, creatine phosphokinase/phosphoglycerate kinase, lactate dehydrogenase and myoglobin.

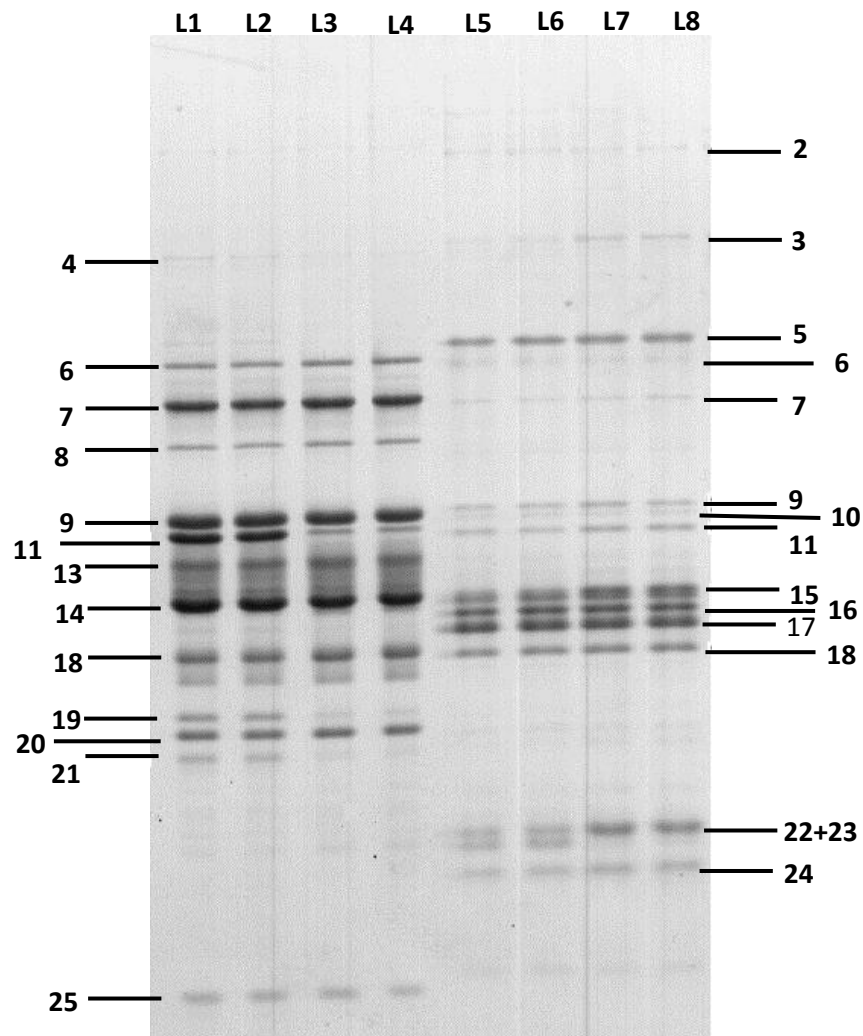


Fig. 4.3 Example of a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation pattern of a soluble sarcoplasmic (lanes 1-4) and myofibrillar (lanes 5-8) protein fraction from the delayed chilling group. 2: C-protein; 3: α -actinin; 4: phosphorylase b/ phosphorylase b kinase; 5: unknown; 6: phosphoglucomutase; 7: pyruvate kinase; 8: phosphoglucose isomerase; 9: enolase; 10: actin; 11: creatin phosphokinase/phosphoglycerate kinase; 13: aldolase; 14: glyceraldehyde phosphate dehydrogenase; 15: troponin T; 16: β -tropomyosin; 17: α -tropomyosin; 18: lactate dehydrogenase; 19: phosphoglycerate mutase; 20: triose phosphate isomerase; 21: myokinase; 22: unknown; 23: troponin I; 24: troponin C; 25: myoglobin

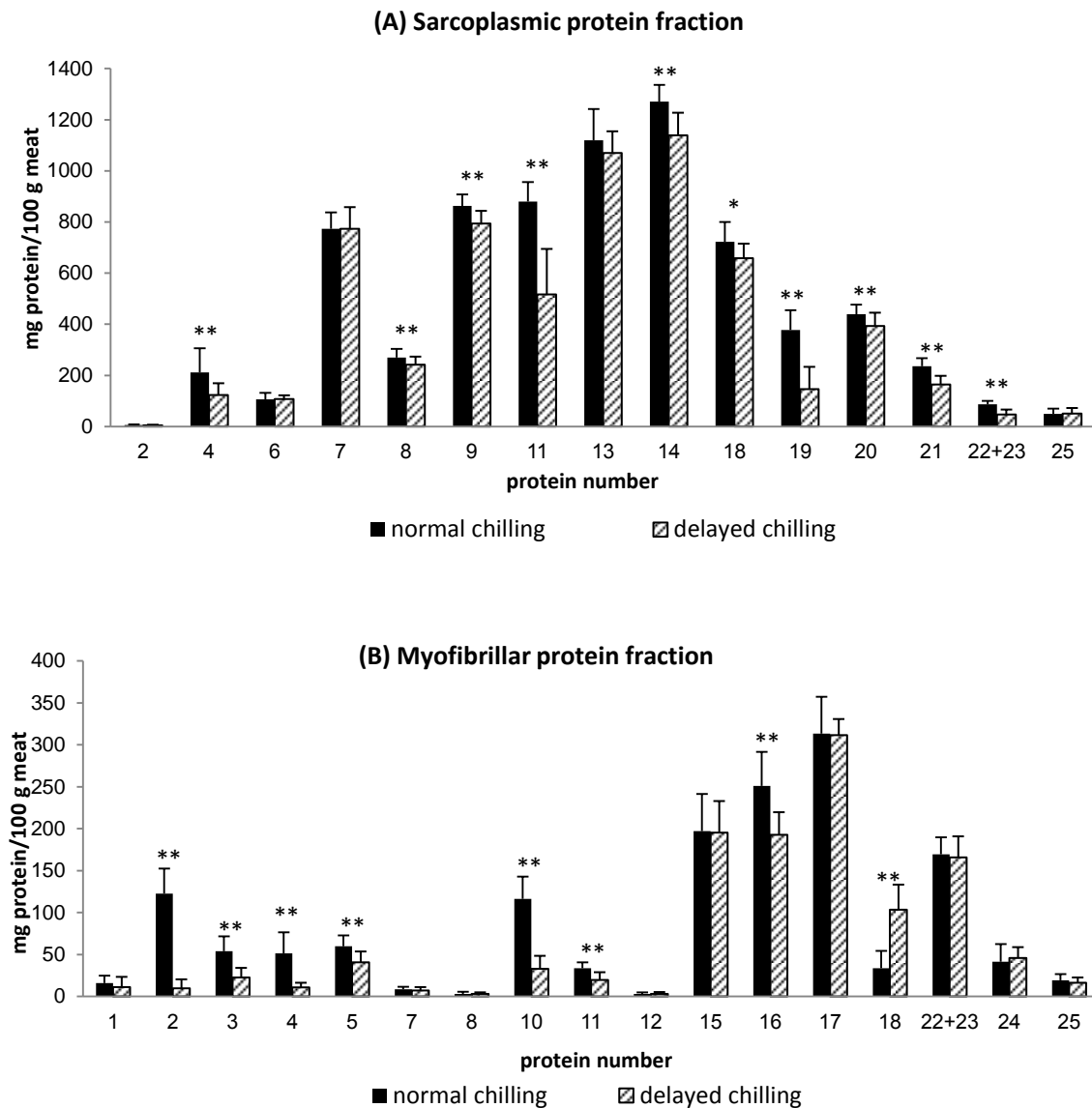


Fig. 4.4 Concentration of soluble sarcoplasmic (A) and myofibrillar (B) proteins separated by SDS-PAGE. 1: M-protein; 2: C-protein; 3: α -actinin; 4: phosphorylase b/ phosphorylase b kinase; 5: unknown; 6: phosphoglucomutase; 7: pyruvate kinase; 8: phosphoglucose isomerase; 9: enolase; 10: actin; 11: creatin phosphokinase/phosphoglycerate kinase; 12: unknown; 13: aldolase; 14: glyceraldehyde phosphate dehydrogenase; 15: troponin T; 16: β -tropomyosin; 17: α -tropomyosin; 18: lactate dehydrogenase; 19: phosphoglycerate mutase; 20: triose phosphate isomerase; 21: myokinase; 22: unknown; 23: troponin I; 24: troponin C; 25: myoglobin. *,**=significant difference at $P<0.05$ and $P<0.01$, respectively. Error bars represent standard deviations

4.3.5 Protein oxidation

The protein carbonyl content was not significantly different between the delayed and normal chilling LD (Table 4.1) for both day 0 and day 8 of display ($P>0.05$). Protein carbonyls increased during the 8 day display period, so there was protein oxidation but this did not differ between chilling treatments. In the present study, there was also no link between lipid and protein oxidation. This is in agreement with Lund et al. (2008) who concluded that there was no simple correlation between lipid oxidation and protein oxidation in high fat and low fat pork patties. Similarly, Lund et al. (2007b) reported that the protein carbonyl content in beef patties did not differ among antioxidant treatments and different packaging atmospheres after 1 day of storage, but lipid oxidation increased markedly in the presence of oxygen in the packaging atmospheres after 1 day of storage. In contrast, other studies found protein oxidation was linked to lipid oxidation in turkey meat (Mercier et al., 1998), beef (Mercier et al., 1995) and in fish (Srinivasan & Hultin, 1995). Rowe et al. (2004) examined the effect of early post-mortem protein oxidation on the colour and tenderness of beef steaks and found that increased protein oxidation of muscle early post-mortem could have negative effects on fresh meat colour and tenderness. Other authors have reported possible effects of protein oxidation on colour deterioration of muscle foods but the precise mechanisms have not been clarified (Estévez & Cava, 2004; Ganhão et al., 2010).

In the current study, increased protein denaturation (lower solubility) in the delayed chilling treatment does not seem to be related to protein oxidation when comparing to the normal chilling treatment. In contrast, Rowe et al. (2004) found a higher total carbonyl content and lower protein solubility in irradiated meat compared to non-

irradiated meat suggesting protein oxidation may ultimately result in a loss of catalytic activity and, increased protein aggregation and loss of solubility.

The reason for the absence of an effect of delayed chilling on lipid and protein oxidation in this experiment whereas there was an effect on colour is not clear. It could be suggested that other endogenous enzymes such as lipase are negatively affected by the high temperature, and that a lower rate of lipolysis may have lead to a lower amount of free fatty acids and lower lipid oxidation in meat. In addition, it can be assumed that the normal chilling in this experiment is slightly faster than what happens in commercial practice because of the smaller size of the cut samples, and that inversely the delayed chilling corresponds to a slower chilling rate. Therefore, more research is warranted on lipid oxidative stability in meat from different chilling regimes. For this purpose, the experiment could be repeated with normal chilling of pork LD on the carcass like in commercial practice.

4.4 CONCLUSION

Delayed chilling of pork LD induced protein denaturation and PSE characteristics, and reduced the activity of the antioxidant enzymes CAT, SOD, and GSH-Px at 24 hours pm. This was accompanied by a lower colour stability during subsequent cooled display, but lipid and protein oxidative stability were not negatively affected.

EFFECT OF HOT BONING ON COLOUR STABILITY, ANTIOXIDANT ENZYME ACTIVITIES AND OXIDATION IN BEEF INNER AND OUTER *M. Biceps femoris*

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EFFECT OF HOT BONING ON COLOUR STABILITY, ANTIOXIDANT ENZYME ACTIVITIES AND OXIDATION IN BEEF INNER AND OUTER *M. Biceps femoris*

ABSTRACT

The aim of this study was to investigate the effect of hot boning on antioxidant enzyme activities, colour stability and, lipid and protein oxidation in inner and outer *M. Biceps femoris* (IBF and OBF) from 7 Belgian Blue young bulls. The L^* value was higher in the cold-boned IBF than OBF ($P<0.01$) resulting in two-toning, whereas colour was not significantly different between hot-boned IBF and OBF. Concomitantly, protein solubility was lower, the decrease in a^* value and the increase in %metmyoglobin over a 7 day display period was higher ($P<0.05$) for the cold-boned IBF compared to other groups. However, there were no significant differences in antioxidant enzyme activities, lipid and protein oxidation at day 10 of display. Hot boning of beef *M. Biceps femoris* has advantages over cold boning, especially for eliminating heat shortening and increasing the colour uniformity in this muscle.

5.1 INTRODUCTION

The muscle tissue and meat quality of double-muscled Belgian Blue cattle differs in many aspects from meat of conventional breeds (Clinquart et al., 1998; De Smet, 2004). The more glycolytic fiber type in combination with the larger muscle mass of these animals results in faster glycolysis and leads to faster pH fall and slower cooling post-mortem (pm) compared to carcasses of non double-muscled and lighter animals. Particularly in the deeper muscles of the hindquarter this may induce heat shortening (pH <6 and temperature >35 °C; Thompson, 2002) and the occurrence of pale and two-toned colour. It was previously reported that heat shortening was at least partly responsible for two-toning in deeper muscles of Belgian Blue beef (De Smet et al., 2008). This defect could potentially be alleviated if a faster rate of cooling of these deeper muscles could be achieved.

During storage, meat is also subject to oxidative deterioration leading to quality degeneration and loss of nutritional value and reduced sensory quality. *In vivo* and in meat, there are several mechanisms to protect oxidative processes including the endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). SOD plays an important role in protecting against damage by superoxide anion radicals, while CAT and GSH-Px are considered to be hydrogen peroxide and fatty acid hydroperoxides decomposing enzymes in the cytosol (Chan & Decker, 1994; Matés et al., 1999; Muller et al., 2007).

In a previous study, we found that lipid oxidation and formation of metmyoglobin were higher in the inner part of the *M. Biceps femoris* compared to the outer part and the *M. Longissimus*, associated with an increased degree of heat shortening and protein

denaturation (Pastsart et al., 2013). The lower oxidative stability of the IBF was also accompanied by reduced activities of CAT and GSH-Px, suggesting that protein denaturation resulting from severe heat shortening also affects the activity of endogenous enzymes. To what extent the reduced antioxidant enzyme activity also contributes to the reduced post-mortem oxidative stability of the muscle remains to be established.

Hot boning is the technique of deboning warm carcasses shortly after slaughter resulting in faster cooling of the cuts and reducing the required cooling capacity compared to the conventional cold boning (Falk et al., 1975). Hot boning is known to affect several meat quality traits, in particular its effect on tenderness (Wheeler et al., 1991). However, the effects of hot boning on the oxidative stability of meat have not been frequently addressed. Sammel et al. (2002) reported that partial hot boning improved the colour stability of beef *M. Semimembranosus*. Therefore, the objective of this study was to investigate the influence of hot boning on the colour and oxidative stability of beef inner and outer *M. Biceps femoris* compared to conventional cooling and cold boning. Additionally, the influence of hot boning of this muscle on the activities CAT, GSH-Px and SOD was examined.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Seven double-muscled Belgian Blue young bulls with mean age at slaughter and carcass weight of 22.7 ± 2.5 months and 495.9 ± 56.3 kg respectively, were used. The animals

originated from commercial farms in Belgium and were slaughtered in two different abattoirs.

5.2.2 Hot boning and meat sampling

Hot boning was performed on the 7 carcasses within 1 hour pm. The BF muscle of one carcass side was completely cut out to allow faster cooling, whereas the other carcass side was left intact and served as control for cold boning. The hot-boned BF muscle was vacuum packed and stored in the dark at 12 °C during the first 5 h pm and thereafter at 4 °C until 48 h pm. Cold boning was performed at 48 h pm. At that time, the outer and the inner *M. Biceps femoris* (OBF and IBF, respectively) were sampled, vacuum packed and frozen at -20 °C until enzyme activity analyses. For the simulated retail display, additional samples were wrapped in oxygen permeable foil and immediately displayed at 4 °C under fluorescent light (approximately 1200 lux).

5.2.3 Temperature and pH measurements

Temperature and pH were measured at 1, 3, 5 and 48 hour pm in the IBF at 10 cm depth and the OBF at 2 cm depth. Conditions for heat shortening were defined as pH lower than 6.0 and temperature higher than 35 °C (Thompson, 2002).

5.2.4 Colour measurements

Colour parameters were measured with a Hunterlab Miniscan colour meter (D65 light source, 10° standard observer, 45°/0° geometry, 1-inch light surface, white standard) at 0, 1, 2, 3, 4, 5, 6, 7 and 10 days of display to assess colour stability (AMSA, 2012). C.I.E. L^* values (lightness), a^* values (redness), and spectral reflectance (400-700 nm) data were collected. The percentage metmyoglobin (%MetMb) was determined using spectral reflectance values at the wavelengths 520, 530, 570, 580 and 700 nm and using the formulas of Krzywicki (1979), as modified by Lindahl et al. (2001). Initial L^* and a^* values at day 0 (d0) after blooming for 2 hours were used to characterize the colour of the meat. The colour stability was evaluated from the average decline in a^* value and the increase in %MetMb during the display from day 1 until day 7, and is expressed as Δa^* and $\Delta\% \text{MetMb}$ respectively. Two-toning in BF, expressed as the difference between the L^* value at d0 between IBF and OBF, was calculated as an index for colour acceptance.

5.2.5 Protein solubility

At day 0 and day 10 of display, protein solubility was measured in a low ionic strength solution (150 mM NaCl) according to Claeys et al. (2002), and was expressed in mg protein per g meat. A lower protein solubility was considered to reflect a higher degree of protein denaturation.

5.2.6 Sarcomere length

Sarcomere length was measured by laser diffraction and light microscopy according to the method described by Vandendriessche et al. (1984) and was expressed in μm .

5.2.7 Lipid and protein oxidation

Lipid and protein oxidation were determined on samples at day 0 and day 10 of display. Lipid oxidation was assessed spectrophotometrically by the thiobarbituric acid reactive substances (TBARS) method based on Tarladgis et al. (1960) and is expressed as μg malondialdehyde (MDA) per g meat.

Protein oxidation was assessed by measuring the carbonyl content of the meat samples (Ganhão et al., 2010). The measurement of protein carbonyls was done by following their covalent reaction with 2,4-dinitrophenylhydrazine (DNPH). This reaction results in the formation of a stable 2,4-dinitrophenyl hydrazone product. Total carbonyl content was quantified spectrophotometrically at 370 nm, using a molar absorption coefficient of $21.0/(\text{mM}\cdot\text{cm})$ and expressed as nmol DNPH incorporated/mg protein by using the formula as described by Jongberg et al. (2011).

$$\frac{C_{\text{hydrazone}}}{C_{\text{protein}}} = \frac{A_{370}}{\epsilon_{\text{hydrazone},370} \cdot (A_{280} - A_{370} \cdot 0.43)} \cdot 10^6$$

Where, $\epsilon_{\text{hydrazone},370}$ is $21000 \text{ M}^{-1}\text{cm}^{-1}$, and $0.43 = \epsilon_{\text{hydrazone},280}/\epsilon_{\text{hydrazone},370}$.

5.2.8 Antioxidant enzyme activity assays

Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) activities were determined on samples at day 0 of display. The muscle samples were kept on ice during the procedure after thawing. A 5 g muscle sample was homogenized in 10 ml of 0.05 M phosphate buffer (pH 7.0) and centrifuged at 4 °C for 20 min at 7000g. The supernatant fraction was filtered through glass wool before determining enzyme activities.

The SOD activity assay was performed as described by Marklund and Marklund (1974) by measuring the inhibition of pyrogallol autoxidation. One unit of enzyme activity was defined as the amount of sample needed to inhibit the reaction by 50%. The activity of GSH-Px was determined by measuring the oxidation of NADPH. One unit of GSH-Px activity was defined as the amount of extract required to oxidize 1 μmol of NADPH per min at 25 °C (Hernández et al., 2004). The CAT activity was determined according to the method of Aebi (1983). One unit of CAT activity was defined as the amount of sample required to decompose 1 μmol of H_2O_2 per min at room temperature.

5.2.9 Statistical analysis

Data in the tables and text are presented as mean values \pm standard deviation. Experimental data were analyzed using a model with the fixed effects of boning method, muscle, slaughter day, and all two-way interactions, and using the General Linear Model (GLM) procedure of SAS® Enterprise Guide®, version 5 (SAS Institute Inc., Cary, NC, USA). Post-hoc tests were performed at a significance level of $P < 0.05$ using the Tukey

correction for multiple tests. For the heat shortening time and two-toning occurrence, a paired t-test was used to compare the mean values between hot and cold boning method.

5.3. RESULTS

5.3.1 Temperature and pH decline

The average pH and temperature decline in IBF and OBF muscles for cold and hot boning treatments are shown in Table 5.1.

At 1 h pm, the pH value in IBF was significantly lower than in OBF for both the hot and cold boning treatments ($P < 0.001$), but there was no significant difference between hot-boned IBF versus cold-boned IBF, and hot-boned OBF versus cold-boned OBF. At 3 and 5 h pm, pH values in cold-boned IBF were lower than for the other treatments ($P < 0.05$). After 48 h pm, the pH was lower in the cold-boned OBF than in the other treatments ($P < 0.001$). It should be noted that there was a higher decrease in pH value for the cold-boned IBF than for the other groups ($P < 0.05$) during the first 5 h pm.

Similarly, the temperature fall at 1h pm was significantly different between the muscles ($P < 0.001$) but there was no significant difference between boning methods. The temperature at 3 and 5 h pm was not significantly different between IBF and OBF for hot-boning method while it was higher in IBF than OBF for cold-boning method.

The duration of heat shortening was estimated at approximately 3.40 hours in the cold-boned IBF, whereas there was no heat shortening in the hot-boned IBF and in the OBF.

Table 5.1 Comparison of pH, temperature and heat shortening time (mean \pm SD) between cold-boned and hot-boned for inner and outer *M. Biceps femoris* (IBF and OBF) muscle (n=7)

	Cold- Boned		Hot- Boned		P-values		
	IBF	OBF	IBF	OBF	Boning method	Muscle	B \times M
pH 1h	6.55 \pm 0.23 ^b	6.89 \pm 0.10 ^a	6.53 \pm 0.21 ^b	6.87 \pm 0.17 ^a	0.583	<.001	0.986
pH 3h	5.79 \pm 0.24 ^c	6.78 \pm 0.17 ^a	6.23 \pm 0.26 ^b	6.66 \pm 0.17 ^a	0.027	<.001	<.001
pH 5h	5.41 \pm 0.04 ^d	6.59 \pm 0.17 ^a	6.10 \pm 0.28 ^c	6.29 \pm 0.36 ^b	0.014	<.001	<.001
pH 48h	5.45 \pm 0.06 ^a	5.36 \pm 0.06 ^b	5.43 \pm 0.06 ^a	5.43 \pm 0.05 ^a	0.261	0.004	<.001
pH 1h-5h	1.14 \pm 0.21 ^a	0.29 \pm 0.15 ^c	0.43 \pm 0.10 ^b	0.58 \pm 0.43 ^b	0.021	<.001	<.001
Temperature 1h	39.21 \pm 0.86 ^a	30.84 \pm 2.79 ^b	39.07 \pm 0.82 ^a	31.13 \pm 2.63 ^b	0.807	<.001	0.438
Temperature 3h	38.44 \pm 0.62 ^a	23.44 \pm 3.10 ^b	18.94 \pm 2.36 ^c	17.37 \pm 2.51 ^c	<.001	<.001	<.001
Temperature 5h	35.99 \pm 0.80 ^a	21.00 \pm 2.29 ^b	12.67 \pm 2.35 ^c	12.46 \pm 1.74 ^c	<.001	<.001	<.001
Temperature 48h	5.67 \pm 1.05	5.03 \pm 0.72	4.77 \pm 0.60	4.77 \pm 0.60	<.001	<.001	<.001
Temperature 1h-5h	3.23 \pm 1.15 ^d	9.84 \pm 2.07 ^c	26.40 \pm 1.72 ^a	18.67 \pm 2.13 ^b	<.001	0.043	<.001
Heat shortening time (h)	3.40 \pm 0.73 ^a		0.00 ^b		<.001	<.001	<.001

^{a, b, c, d} Within a row, mean values with different superscripts differ significantly at P<0.05

5.3.2 Colour stability

The evolution of L^* values throughout day 1 to day 10 of display is illustrated in Fig. 5.1. During 10 days of display the L^* value slightly increased in all muscles from two different methods and it was the most significant for the cold-boned IBF muscle ($P<0.01$). The average initial L^* values (d0) for the cold-boned IBF was significantly higher compared to others ($P<0.01$). This illustrates the problem of two-toning in the BF muscle from cold boning method. On the other hand, there was no difference in average L^* value between hot-boned IBF and cold-boned OBF muscle. This indicates the problem of two-toning in this muscle could be solved by hot boning method.

The average initial a^* values for the cold-boned IBF was significantly higher than other groups ($P<0.01$). Conversely, after 10 days of display, the a^* value for cold-boned IBF tended to be lowest from others. The change in average a^* values during day 1 to day 10 of display is illustrated in Fig. 5.2.

However, the average %MetMb at day 0 and day 10 of display did not differ for the IBF and OBF in both boning methods. The change in %MetMb throughout day 1 to day 10 of display is shown in Fig. 5.3.

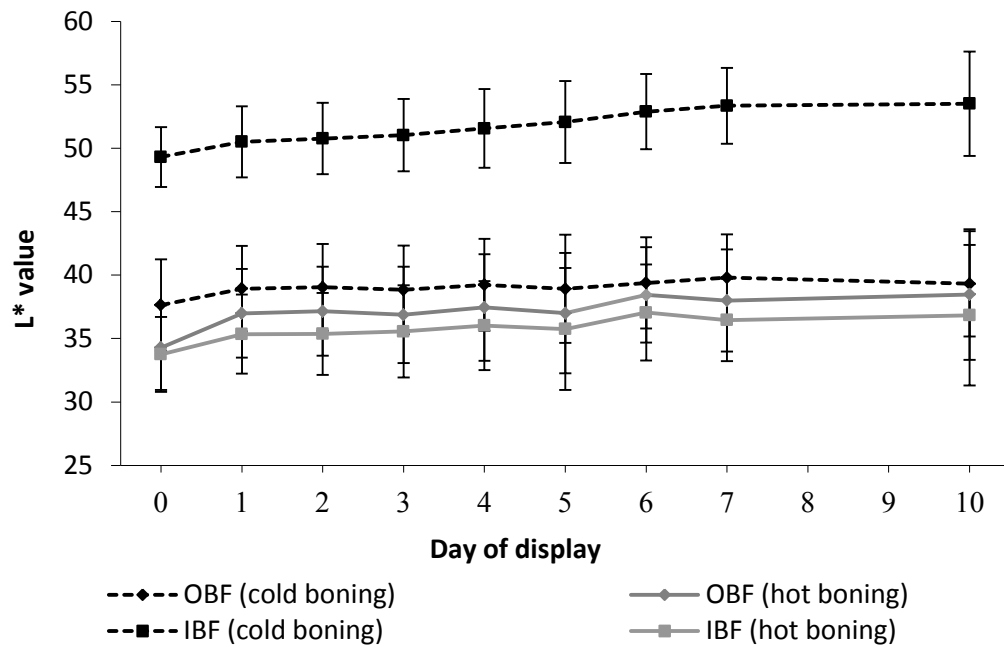


Fig. 5.1 The change in average lightness (L^*) value for inner and outer *M. Biceps femoris* (IBF and OBF) muscle during display ($n=7$). Error bars represent standard deviations

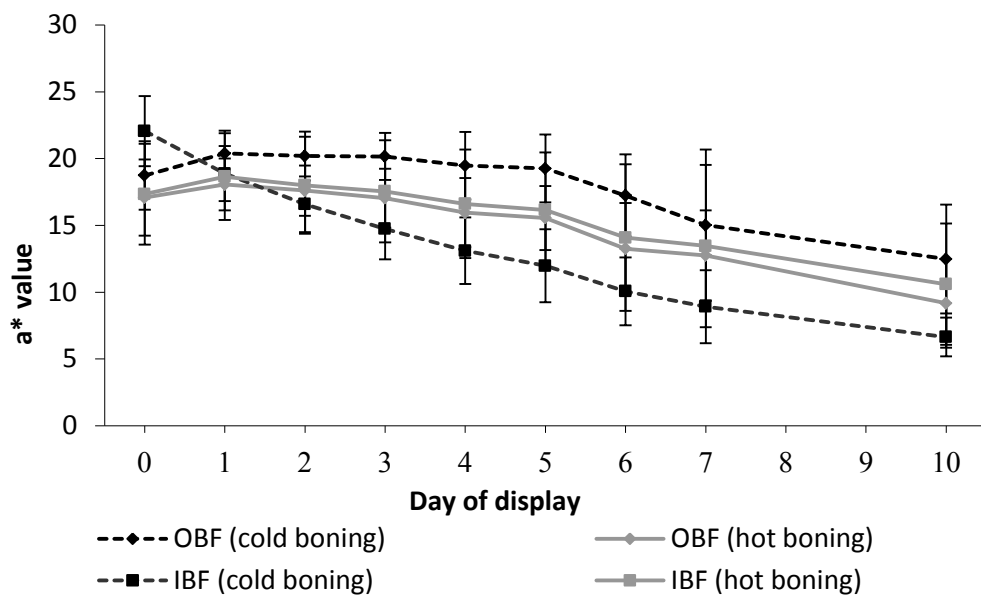


Fig. 5.2 The change in mean redness (a^*) value for inner and outer *M. Biceps femoris* (IBF and OBF) muscle during display ($n=7$). Error bars represent standard deviations

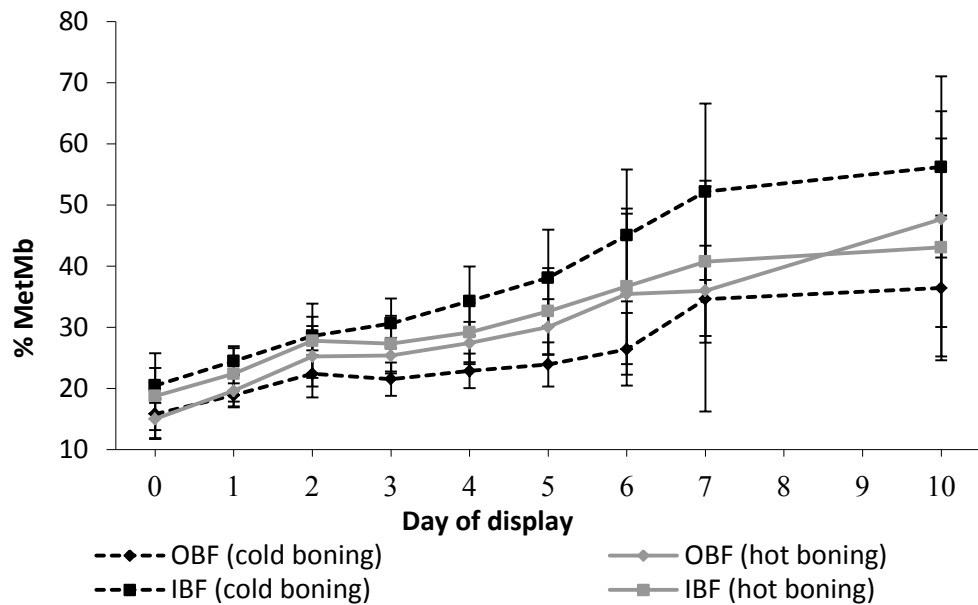


Fig. 5.3 The change in mean %MetMb for inner and outer *M. Biceps femoris* (IBF and OBF) muscle during display (n=7). Error bars represent standard deviations

5.3.3 Sarcomere length

Sarcomere length for cold-boned OBF was higher than hot-boned IBF but not different from cold-boned IBF and hot-boned OBF ($P>0.05$) as shown in table 5.2

5.3.4 Lipid and protein oxidation and protein solubility

TBARS values at day 0 of display were higher in the hot-boned IBF than hot and cold-boned OBF ($P<0.001$), while the values at day 10 of display and the increase in TBARS value after 10 days of storage were not significantly different between muscles and boning methods ($P>0.05$) as shown in Table 5.2.

Table 5.2 Comparison of antioxidant enzyme activities (Unit¹/g), colour stability parameters, lipid oxidation (TBARS), protein solubility (mg protein/g meat) and sarcomere length (mean \pm SD) between cold-boned and hot-boned for inner and outer *M. Biceps femoris* (IBF and OBF) muscle (n=7)

Traits	Cold- Boned		Hot- Boned		P-values		
	IBF	OBF	IBF	OBF	Boning method	Muscle	B×M
CAT d0 (U/g)	93.93 \pm 24.98	107.23 \pm 43.60	93.00 \pm 21.27	104.42 \pm 32.89	0.929	0.324	0.832
GSH-Px d0 (U/g)	1.20 \pm 0.56	1.37 \pm 0.57	1.21 \pm 0.45	1.30 \pm 0.54	0.917	0.477	0.946
SOD d0 (U/g)	70.60 \pm 8.55	62.72 \pm 9.20	71.01 \pm 7.39	66.45 \pm 8.43	0.660	0.095	0.618
Δa^* d1-d7 (/d)	-1.63 \pm 0.38 ^b	-0.82 \pm 0.72 ^a	-0.89 \pm 0.53 ^a	-0.93 \pm 0.53 ^a	0.084	0.034	0.029
$\Delta\%$ MetMb d1-d7 (/d)	4.41 \pm 2.18 ^a	2.06 \pm 2.17 ^b	2.79 \pm 1.65 ^{ab}	2.65 \pm 1.33 ^{ab}	0.434	0.049	0.057
2-toning d0	11.66 \pm 3.25 ^a		0.40 \pm 0.48 ^b		<.001	-	-
TBARS d0 (μ g/g)	0.29 \pm 0.11 ^{ab}	0.21 \pm 0.08 ^b	0.28 \pm 0.18 ^a	0.21 \pm 0.07 ^b	0.748	0.001	0.615
TBARS d10 (μ g/g)	3.20 \pm 2.44	2.06 \pm 1.45	2.20 \pm 2.14	2.69 \pm 1.91	0.882	0.698	0.348
TBARS d10-d0 (μ g/g)	2.92 \pm 2.35	1.85 \pm 1.40	1.92 \pm 2.06	2.48 \pm 1.87	0.874	0.770	0.333
Protein solubility d0 (mg protein/g)	58.15 \pm 5.38 ^b	77.20 \pm 8.84 ^a	79.10 \pm 3.46 ^a	78.44 \pm 5.01 ^a	0.001	0.006	0.003
Protein solubility d10 (mg protein/g)	51.98 \pm 5.16 ^b	66.42 \pm 6.41 ^a	72.51 \pm 10.12 ^a	72.10 \pm 9.90 ^a	<.001	0.014	0.011
Sarcomere length (μ m)	1.81 \pm 0.09 ^{ab}	1.91 \pm 0.18 ^a	1.71 \pm 0.21 ^b	1.76 \pm 0.17 ^{ab}	0.012	0.167	0.347

^{a, b, c, d} Within a row and column mean values with different superscripts differ significantly at $P < 0.05$. ¹ One unit of SOD was defined as the amount of extract needed to inhibit the rate of oxidation by the control (no SOD) by 50%. One unit of GSH-Px activity was defined as the amount of extract needed to oxidise 1 μ mol of NADPH per min at 25 °C. One unit of CAT was defined as the amount of extract needed to decompose 1 μ mol of H₂O₂ per min at room temperature

The protein carbonyls increased approximately 8 fold from the beginning to day 10 of display (Fig. 5.4). At day 0 of display, protein carbonyls for hot-boned OBF was lower than for the other treatments ($P<0.001$), but at day 10 of display there was no significant difference in protein carbonyls among all treatments.

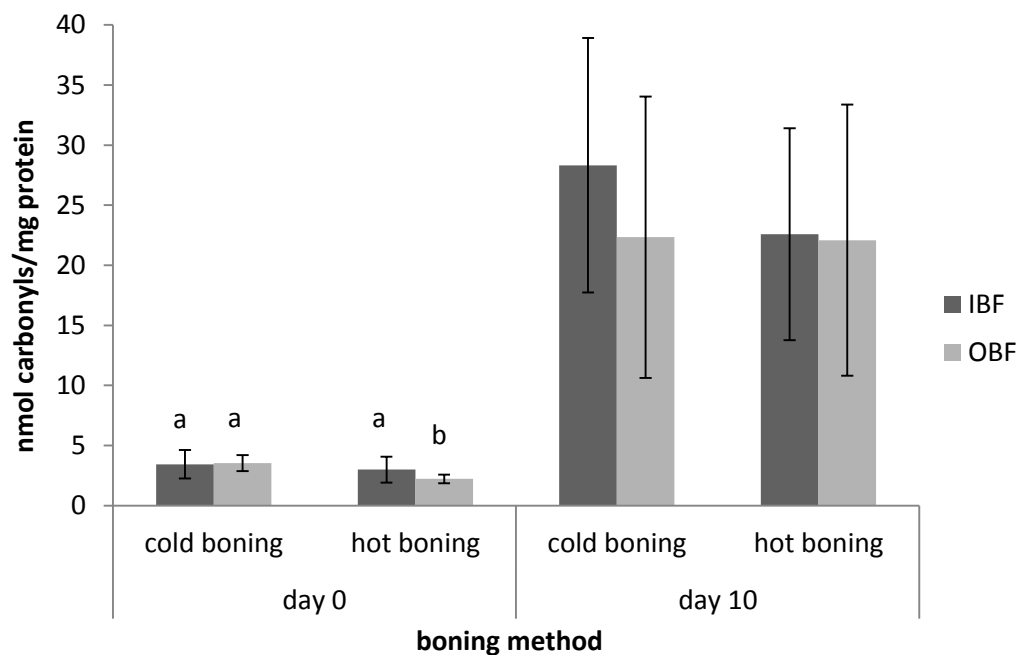


Fig. 5.4 Protein oxidation (nmol carbonyls) for inner and outer *M. Biceps femoris* (IBF and OBF) muscle between hot and cold boning methods ($n=7$). Error bars represent standard deviations. ^{a,b}Mean values at day 0 of display with a different superscripts are significantly different at $P<0.01$

Protein solubility at day 0 and 10 of display was lower in the cold-boned IBF compared to the OBF and hot-boned IBF ($P<0.05$; Table 5.2), suggesting more protein denaturation in the cold-boned IBF resulting from heat shortening.

5.3.5 Antioxidant enzyme activities

The activities of antioxidant enzymes at day 0 of display, expressed in units per gram of meat, are shown in Table 5.2. The results showed that there was no significant difference in activity of SOD, CAT and GSH-Px between muscles and boning methods ($P>0.05$).

5.4 DISCUSSION

The faster pH fall and slower temperature decline in the cold-boned IBF induced heat shortening, which resulted in a paler colour compared to the OBF and thus resulted in an unattractive two-toning appearance of this muscle. Many previous studies have shown that in beef or pork, a lower pH and higher temperature early post-mortem are associated with paler colour and lower water-holding capacity, although quality defects are much less severe for beef than for pork (Fischer & Hamm, 1980; Warris & Brown, 1987; Fernández et al., 1994; Steen et al., 1997). To tackle this problem, hot boning was applied which allowed a faster cooling. The results showed that the temperature and the pH decline were comparable in the hot-boned IBF and OBF. As a consequence, there was no period of heat shortening in the IBF when hot boned.

The large difference in lightness between the IBF and OBF muscle following cold boning was significantly reduced after hot boning and thus also the problem of two-toning. Concomitantly, the colour stability of the IBF muscle was improved when hot boned, as was apparent from the lower decrease in redness and the lower increase in %MetMb during display. Avoiding heat shortening through hot boning does not only reduce the

pale meat appearance, it also improves the colour stability. The common underlying mechanism might be a reduced protein denaturation. Moreover, protein solubility in cold-boned IBF at day 0 and 10 of display was lower than in the other groups. It should be mentioned that protein solubility was measured in a low-ionic strength buffer so that mainly sarcoplasmic proteins and enzymes are covered by this method. A more intense protein denaturation resulting from a faster post-mortem pH decline was suggested to be the cause of reduced activity of several proteolytic and lipolytic enzymes in pork (Claeys et al., 2001).

The SOD, CAT and GSH-Px activities were remarkably similar for the hot- and cold-boned samples. Although there was a tendency for differences between IBF and OBF, it might thus be concluded that the boning method has no effect on the activities of these three endogenous antioxidant enzymes. Consistently, there was no effect of boning method on lipid and protein oxidation at day 10 of display.

5.5 CONCLUSION

The fast pH fall, slow temperature decline and the severe heat shortening in the IBF muscle from double-muscled Belgian Blue bulls under normal cooling practices, resulted in a pale colour and two-toning in the BF. Hot boning of the BF muscle can be seen as an appropriate solution for faster chilling and tackling problems with colour stability. However, hot boning of beef carcass has no significant effect on the CAT, SOD, and GSH-Px, as well as lipid and protein oxidation in meat.

GENERAL DISCUSSION

Oxidative processes, i.e. lipid, pigment and protein oxidation, are major factors limiting the quality and acceptability of meats and meat products. Reactive oxygen species (ROS) generation has been implicated in these oxidative processes but the importance of the antioxidant enzymes in retarding oxidative deterioration of muscle post-mortem is still insufficiently known.

This PhD research was conducted in an attempt to investigate the potential role of antioxidant enzymes (SOD, CAT and GSH-Px) against oxidative processes in meat by using specific inhibitors of the activity of these enzymes. Also, we investigated the effect of muscle type and post-mortem rate of pH and temperature fall, as well as, the boning method on these antioxidant enzymes activities in relation to the meat oxidative stability. The objective of this chapter is to link the findings from the different experimental chapters and to formulate overall conclusions and prospects.

6.1 THE ROLE OF ANTIOXIDANT ENZYMES IN MEAT

In **chapter 2**, mercaptosuccinate ($C_4H_6O_4S$), sodium azide (NaN_3) and diethyldithiocarbamate (DDC) were used as inhibitors for GSH-Px, CAT and SOD, respectively and allowed us to elucidate the function of these antioxidant enzymes in muscle post-mortem. The results showed that mercaptosuccinate inhibited the

activity of GSH-Px but had no effect on SOD and CAT activities. NaN_3 inhibited the activity of CAT but had no effect on GSH-Px and SOD activities. A high concentration of DDC (1 mM) inhibited SOD activity and slightly inhibited CAT but had no effect on GSH-Px. Hence, it was concluded that GSH-Px and CAT could be specifically inhibited for the purpose of the present study, but we could not specifically inhibit SOD (Table 6.1).

Table 6.1 Specific inhibition of enzyme activities

	Mercaptosuccinate	NaN_3	DDC
GSH-Px	↓	Not affected	Not affected
SOD	Not affected	Not affected	↓
CAT	Not affected	↓	↓

With regard to the inhibition mechanism of GSH-Px by mercaptosuccinate, it is known that mercaptosuccinate rapidly binds to the active site selenium of GSH-Px in competition with GSH (Chaudiere et al., 1984), leading to the deactivation of GSH-Px. NaN_3 inhibits catalase by reacting with the heme prosthetic group of the enzyme (Pradhan et al., 2000). Cocco et al. (1981) proposed for the mechanism of inhibition of SOD by DDC that DDC binds to Cu at the active site of CuZn-SOD resulting in elimination of the catalytic activity. The reaction is a cooperative binding of two DDC molecules per Cu atom with consequent removal of the metal from the enzyme.

The potential role of the three antioxidant enzymes on meat oxidative stability is summarized in Table 6.2. However, the effect of CAT and SOD on colour stability could

not be assessed properly since the colour L^* value was strongly affected by the addition of NaN_3 and DDC, respectively.

Table 6.2 Summary of the effect of inhibition of antioxidant enzyme activities on beef oxidative stability

	Inhibited by inhibitors		
	GSH-Px	CAT	SOD
Lipid oxidation	↑	↑	↓
Protein oxidation	↑	Not affected	↓
Myoglobin oxidation	↑	N/A	N/A

N/A: not applicable

The inactivation of GSH-Px by mercaptosuccinate increased TBARS values and protein carbonyls content, and resulted in an enhanced fall of the colour a^* value (red colour). This suggests that GSH-Px is involved in reducing myoglobin as well as lipid and protein oxidation. In this study, lipid, protein and myoglobin oxidation seemed to be linked. A similar finding on the relationship between GSH-Px and lipid oxidation was reported in mouse and human sperm, whereby the inactivation of GSH-Px by mercaptosuccinate had an effect on the rate of lipid peroxidation (Alvarez & Storey, 1989). These authors concluded that the key intermediate in spontaneous lipid peroxidation is lipid hydroperoxide generated by a chain reaction initiated by and utilizing superoxide. Removal of this hydroperoxide by GSH-Px protects cells against peroxidation;

inactivation of the peroxidase allows lipid hydroperoxide to increase and so increases the peroxidation rate.

The inhibition of CAT by NaN_3 resulted in higher TBARS values, but had no significant effect on protein carbonyls content (Table 6.2). The reason for the ineffectiveness of the CAT in inhibiting protein oxidation is unclear. It is possible that catalase alone is insufficient to reduce the substrate (H_2O_2) in muscle, allowing H_2O_2 to interact with metmyoglobin to produce ferrylmyoglobin, which can initiate protein oxidation. When CAT works together with GSH-Px, it may be able to control H_2O_2 concentrations in the meat samples. H_2O_2 was reported to react rapidly with metmyoglobin to form ferrylmyoglobin radicals (Kanner & Harel, 1985). Besides ferrylmyoglobin radicals, highly reactive hydroxyl radicals could have been formed from the H_2O_2 in meat, through Haber-Weiss/Fenton reaction (Johnson et al., 1992).

For the DDC inhibitor, the lower activity of SOD in beef decreased TBARS values and protein carbonyls (Table 6.2). These results suggest that SOD might be not involved in reducing lipid and protein oxidation in muscle post-mortem. The reason for this unexpected opposite finding is unclear. However, these results confirm the relationship between lipid and protein oxidation but both processes seem not to be inhibited by SOD activity.

In the present study, the oxidative processes seemed to be linked. Several studies described the relationship between lipid, myoglobin and protein oxidation in meat (Chaijan, 2008; Faustman et al., 2010; Estévez, 2011). Collectively, during oxidation of oxymyoglobin, both $\text{O}_2^{\bullet-}$ and H_2O_2 are produced and further react with iron to produce HO^{\bullet} which has the ability to penetrate into the hydrophobic lipid region and then

promotes lipid oxidation. The reaction between H_2O_2 and metmyoglobin results in the formation of two active hypervalent myoglobin species; perferrylmyoglobin and ferrylmyoglobin, which also have been found to initiate lipid and protein oxidation. Lipid oxidation results in a wide range of aldehyde products, which are reported to induce the oxidation of oxymyoglobin (Faustman et al., 2010). Moreover, lipid-derived reactive oxygen species, such as peroxy radicals (ROO^\bullet) are the potential initiators of protein carbonylation (Estévez, 2010).

6.2 TIME EFFECTS ON ACTIVITY OF ANTIOXIDANT ENZYMES

In **chapter 2**, across the control groups, it was found that the activity of GSH-Px significantly decreased in time in the order day 0 > day 12 > day 6, whereas, CAT and SOD activities were significantly lower at day 6 and 12 of display compared to day 0, with no difference between day 6 and 12 of display. This corresponds with the results from **chapter 3**, in which after 10 days of display, the activity of CAT and SOD was lower when compared with day 0 of display in the three muscles. Contrary to the results obtained with CAT and SOD activities, there was an overall significant increase in GSH-Px activity at day 10 compared to day 0 of display. Renerre et al. (1996) found that GSH-Px and SOD activities in different beef muscles decreased until day 6 post-mortem. However, these authors did not find a decrease in CAT activity with time. Also Pradhan et al. (2000) reported that CAT activity in beef was stable during 6 days of cold storage, which is in contrast to our results. One might expect a decrease in enzyme activity with time of storage of meat, which then parallels the decrease in meat oxidative stability with time and supports the role of antioxidant enzymes in retarding oxidative

deterioration. Our findings and the literature information, however, does not show a consistent decline for all antioxidant enzymes with time. This illustrates that meat oxidative stability is not only determined by the activity of antioxidant enzymes.

6.3 THE EFFECT OF MUSCLE TYPE, PH AND TEMPERATURE FALL ON ACTIVITY OF ANTIOXIDANT ENZYMES IN RELATION TO OXIDATIVE STABILITY IN MEAT

In **chapter 3**, the effect of muscle type and, pH and temperature fall on antioxidant enzyme activities and oxidative stability was investigated in three different muscle types namely, inner *M. Biceps femoris* (IBF), outer *M. Biceps femoris* (OBF) and *M. Longissimus dorsi* (LD). It is well known that a more glycolytic muscle fibre type and heavier carcasses compared to conventional animals results in a faster pH decline and slower temperature fall post-mortem, particularly in the deeper layer of the large muscle such as *M. Biceps femoris* (BF) (Clinquart et al., 1998; De Smet, 2004). This may induce heat shortening and result in a paler colour in the deeper layer in this muscle, accompanied by two-toning (De Boever et al., 2009; Sammel et al., 2002). Heat shortening leads to increased protein denaturation and tougher meat (Locker & Daines, 1975). The results showed that the duration of heat shortening was longer in IBF than in LD. The higher temperature and lower pH condition in the IBF had a negative effect on GSH-Px and CAT activity but SOD was not affected. In consequence, IBF decreased more in red colour (Δa^* value) during the first 7 days of display and a higher %metmyoglobin compared to the other two muscles. In line with this, the TBARS values at day 10 of display were significantly higher in the IBF than the OBF and LD. The lower GSH-Px and CAT activities were associated with lower colour and oxidative stability of the IBF. This fits with the results obtained in

chapter 2, in which inhibiting the GSH-Px and CAT activities concomitantly increased lipid oxidation and inhibiting the GSH-Px activity also increased the initial formation of metmyoglobin.

In **chapter 4**, delayed chilling of pork *M. Longissimus dorsi* (LD) resulted in a lower activity of CAT, SOD and GSH-Px at 24 hours after cutting compared to normal chilling. The delayed chilling also induced PSE characteristics, heat shortening, lower protein solubility and lower concentration of individual proteins in both the sarcoplasmic and myofibrillar fraction compared to normal chilling but lipid and protein oxidation were not affected. Since no meaningful lipid and protein oxidation was observed, one cannot conclude on the role of the antioxidant enzymes in protecting against oxidation in this experiment.

6.4 THE EFFECT OF HOT BONING ON ANTIOXIDANT ENZYMES AND OXIDATIVE STABILITY IN MEAT

The combination of high temperature and fast pH decline in the cold-boned IBF induced heat shortening and lead to a paler colour compared to the OBF and thus in an unattractive two-toning appearance of BF muscle. To solve this problem, hot boning was applied which allowed a faster temperature fall. The results in **chapter 5** showed that the temperature and the pH decline were comparable in the hot-boned IBF and OBF. As a consequence, there was no period of heat shortening in the IBF when hot boned. Additionally, the large difference in lightness between the IBF and OBF was reduced after hot boning and no two-toning occurred. Concomitantly, the colour stability of the

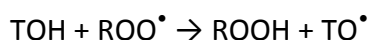
IBF muscle was improved when hot boned, as was apparent from the lower decrease in redness and the lower increase in %MetMb during display. These results were similar to the work of Sammel et al. (2002) who investigated the influence of pre-rigor temperature and pH on muscle chemistry, initial colour and colour uniformity of the inside *M. Semimembranosus* (ISM) and outside *M. Semimembranosus* (OSM). The results showed that cold-boned ISM had a slower chill rate and faster pH decline, less metmyoglobin reducing ability, oxygen consumption, water holding capacity and colour stability than the OSM. Cold-boned steaks were 2-toned in colour and visually unacceptable at d 3 of display. Hot-boned ISM and OSM chilled at the same rate and had similar pH declines, similar chemical characteristics and uniform stable colour for 5 d of display, thus chilling beef ISM faster can produce uniform stable colour.

In this study, the SOD, CAT and GSH-Px activities were not significantly different between cold and hot boning treatments. Concomitantly there was no effect of boning method on lipid and protein oxidation during display. The reason why in this study no difference in activity of enzymes was observed is not clear. The period of heat shortening in the cold-boned IBF in this experiment was only slightly shorter than in the cold-boned IBF in **chapter 3** (3.4 versus 3.9 hours respectively), so large differences in the effect of heat shortening on the enzyme activities are unlikely. On the other hand, when comparing the muscles across boning methods, the CAT and GSH-Px activities tended to be lower and the SOD activity tended to be higher in IBF compared to OBF. This is in line with the results in **chapter 3**.

6.5 FUTURE PROSPECTS

The current study has added insight in the role of antioxidant enzymes (GSH-Px, CAT and SOD) in maintaining the oxidative stability of muscle post-mortem. However, more research is warranted to estimate the role and contribution of the post-mortem activity of other endogenous antioxidants in protecting meat against oxidative processes relative to these 3 enzymes. Besides GSH-Px, CAT and SOD, it is known that other intracellular enzymes, such as glutathione *S*-transferase (GST) protects cells from oxidative stress by catalyzing nucleophilic attack by reduced glutathione (GSH) on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulphur atom (Mannervik & Danielson, 1988; Hayes et al., 2005). Additionally, they can reduce lipid hydroperoxides through their Se-independent glutathione peroxidase activity and these enzymes can also detoxify lipid peroxidation end products such as 4-hydroxynonenal (Sharma et al., 2004). NADH cytochrome-*b*₅ reductase can be another relevant enzyme for further study because it is involved in the reduction of metmyoglobin in meat (metmyoglobin reducing ability) (Arihara et al., 1995). Also, ferritin (Torti & Torti, 2002) and other storage and transport proteins (e.g. transferrin, lactoferrin, haptoglobin, caeruloplasmin, metallothionein (Thurnham, 1990) and carnosine (Chan & Decker, 1994)) sequester transition metals in forms which cannot catalyse the conversion of O₂^{•-} and H₂O₂ to the more damaging HO[•] (Halliwell *et al.*, 1995). Another important protective mechanism is called chain-breaking antioxidants. Plenty of work has been carried out on vitamins E, C and β-carotene, as well as ubiquinol-10, thiols and uric acid, that are all capable of interrupting free-radical chain reactions (Stocker et al., 1991). Vitamin E (α-tocopherol, TOH) is the most important of these compounds in plasma because it is present in

concentrations at least 15 times higher than the other compounds (Burton et al., 1983). It is also an essential component of cell membranes. When it encounters a peroxy radical (ROO^\bullet), TOH donates a hydrogen from its chromanol phenolic group to form a hydroperoxide and a tocopheroxyl radical (TO^\bullet) (Packer, 1993):



Numerous studies have demonstrated that these antioxidants protect cells from oxidative stress *in vivo*. The role of some of these compounds in retarding oxidative processes in muscle post-mortem is well described, e.g. for vitamin E (Buckley et al., 1995; Faustman et al., 1998), but for others this is unknown. Therefore, the function and the relative contribution of different exogenous antioxidants needs further investigation in muscle foods, particularly in relation to the function of GSH-Px, CAT and SOD. E.g. it is known that dietary polyphenols interact with and affect the activity of antioxidant enzymes *in vivo* (Khan et al., 1992; Frei & Higdon, 2003; Koren et al., 2010). It would therefore be interesting to examine these interactions also in muscle post-mortem.

Exploring other factors and sources of variation in these enzyme activities is also warranted. It is well known that there are significant differences in meat quality among different muscles and breeds. In the present study, the post-mortem energy metabolism was investigated in this respect. Other factors might also be worthwhile investigating. Meat with a higher intramuscular fat (IMF) content has a better flavour (Lu et al., 2008). However, meat with a higher lipid content is also more susceptible to attack by ROS and formation of lipid hydroperoxides. Backfat tissue is potentially more predisposed to lipid peroxidation due to its higher concentration of polyunsaturated fatty acids.

Consequently, one could hypothesize that muscles and animal types higher in fat content have evolved towards a higher activity of antioxidant enzymes as a protective mechanism. This remains to be elucidated. Comparing the results for beef and pork from the different experiments in this study also revealed large differences. The CAT activity was approximately 18 fold higher in pork LD than in beef LD, whereas GSH-Px and SOD activities were 2-3 fold higher in beef LD than in pork LD. This illustrates that there is a great variation in antioxidant enzyme activities among species.

Retrospective attention could be paid to the experimental design and methodologies used in this study, and limitations could be remediated in follow-up studies. For example, the determination of lipid oxidation products was limited to TBARS analyses. This method has been criticized for lack of specificity, although it is widely used. Other secondary oxidation products present in meat, e.g. hexanal, could also be determined. The analysis of primary oxidation products through the determination of the peroxide value at several time points during display could also be of value. The comparison of the evolution of primary and secondary oxidation products throughout the shelf life could shed more light on the process of lipid oxidation and the role of antioxidant enzymes therein. The choice of the method for simulating retail display could also be varied. We used a protocol of exposure to ambient atmosphere, but nowadays meat is frequently packaged in high O₂ atmosphere (70-80% O₂ with 7-10 days shelf life) and in these conditions we could expect a different evolution of oxidative process. Therefore, further research could investigate the role of the same and other enzymatic antioxidants and non-enzymatic compounds involved in the oxidative stability of meat, by determining primary and secondary products of oxidation after repeated sampling during display of

meat in different modified atmosphere packages compared to normal display. The period of the display could be shorter, i.e. from 10-12 days to 4-6 days, since the turnover of meat in commercial conditions is faster than the display periods we installed in the present study. The interval periods for evaluating the oxidative stability could be reduced to sampling every day or every 2 days for a better understanding of the evolution of oxidation processes in meat.

Much research related to antioxidant enzymes is focusing on molecular cloning and characterization. In consequence, little is known about the gene expression and regulation of antioxidant systems in meat. Transcriptomics and proteomics are new technologies that may help to identify molecular markers of meat quality. Although several studies reported the expression of antioxidant enzymes in various tissues from different species (Yuan et al., 1996; Xu et al., 2007; Moreira et al., 2011), it is currently not known whether the expression of these genes is correlated with meat quality, and whether these genes are indirect markers of meat quality. More research is warranted on the differences in the expression of antioxidant genes in meat from different species and the regulatory mechanisms of the gene expression occurring in different tissues of animals need to be defined. Therefore, the relationship between mRNA expression and meat quality needs further investigation. Genetic variants for antioxidant enzymes have been detected in humans and are associated with diseases (Rahman et al., 2004; Bentley et al., 2008), but antioxidant enzyme gene polymorphisms in farm animal species in relation to meat quality have not been described. The results from association analyses may be useful for exploring the potential of antioxidant enzymes genetic variants as genetic markers in livestock breeding. The use of such genetic markers could not only be

helpful for improving meat quality but also to improve the endogenous antioxidant status of farm animals *in vivo*.

Oxidation of lipid, myoglobin and protein is one of the key factors limiting the quality and acceptability of muscle foods. Hence, lowering these oxidations can enhance the shelf-life stability of meat and meat products. The living animal possesses antioxidant defense mechanisms, among which the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). However, the importance of these antioxidant enzymes in retarding oxidative processes in muscle post-mortem is still not well known. Therefore, the main purpose of this PhD research was to investigate the potential role of antioxidant enzymes (SOD, CAT and GSH-Px) in protecting meat from oxidative processes by using specific inhibitors of the activity of the enzymes. Also, we investigated the effect of muscle type and post-mortem rate of pH and temperature fall, as well as, the chilling method on activity of these antioxidant enzymes in relation to oxidative stability in meat and meat quality.

Chapter 1 consists of a **literature review**, describing the background on oxidative stress and antioxidant defense mechanisms in the living animal. An overview of meat quality and oxidation processes in meat is also presented.

In the experiments presented in **chapter 2**, the role of GSH-Px, CAT and SOD enzymes was investigated in relation to oxidative processes in beef through inhibition of their post-mortem activities. Mercaptosuccinate, sodium azide (NaN_3) and diethyldithiocarbamate (DDC) were used as specific inhibitors for GSH-Px, CAT

and SOD, respectively, at three different concentrations versus a control. The results showed that the activity of GSH-Px was reduced whereas TBARS values (lipid oxidation) and protein carbonyls (protein oxidation) were increased with increasing concentrations (up to 0.4 mM) of mercaptosuccinate. The CAT activity was completely inhibited and TBARS values were increased at concentrations of 0.03% and 0.06% of NaN_3 compared to lower concentrations, but protein carbonyls were not affected. The activity of SOD, TBARS values and protein carbonyls were reduced at the highest concentration of DDC (1 mM). It was concluded that GSH-Px and CAT may have an important role in retarding lipid oxidation in meat.

Chapter 3 focused on the effect of muscle types, inner and outer *M. Biceps femoris* (IBF and OBF respectively) and *M. Longissimus dorsi* (LD), and the rate of pH and temperature fall post-mortem on the activities of SOD, CAT and GSH-Px during simulated retail display in relation to meat quality parameters such as colour, lipid oxidation and metmyoglobin formation in double-muscled Belgian blue beef ($n = 40$). The results showed the activities of CAT and GSH-Px were lower, whereas lipid oxidation and formation of metmyoglobin were higher in the IBF compared to the OBF and the LD, associated with an increased degree of heat shortening and protein denaturation. The lower CAT and GSH-Px activities in the IBF might result from increased denaturation induced during the longer heat shortening time. However, heat shortening did not affect the SOD activity and the α -tocopherol content. The relationships between the enzyme activities and meat quality traits observed in this study suggest that these endogenous antioxidant enzymes are implicated in the protection against oxidative damage in meat during cold storage.

Chapter 4 aimed to investigate the influence of high post-mortem temperature on the activities of antioxidant enzymes and meat quality in pork *M. Longissimus dorsi* (LD). Pork LD was sampled from the left and right side of 15 pig carcasses at 30 min post-mortem. The LD of one carcass side was kept at 40 °C in an hot air oven for 4 hours and then moved to a chiller at 4 °C until 24 hours pm (delayed chilling), whereas the other side was immediately placed in the same chiller (normal chilling). Delayed chilling resulted in PSE characteristics, heat shortening, lower protein solubility and the activities of CAT, SOD, and GSH-Px at 24 hours after cutting compared to the normal chilling treatment. Colour L^* values were higher during 8 days of display and a^* values were significantly lower from day 4 of display on in the delayed compared to the normal chilling treatment. TBARS values were lower after 8 days of display in the delayed chilling treatment, but no difference in protein carbonyls was noted. It was concluded that delayed chilling of pork LD has a negative effect on the activity of the antioxidant enzymes which was accompanied by a lower colour stability, but lipid and protein oxidative stability was not negatively affected.

In **chapter 5**, the effect of hot boning on antioxidant enzyme activities, colour stability, and, lipid and protein oxidation in the IBF and OBF muscles from 7 Belgian Blue young bulls was investigated. The L^* value was higher in the cold-boned IBF than OBF resulting in two-toning, whereas colour was not significantly different between hot-boned IBF and OBF. Concomitantly, protein solubility was lower, the decrease in a^* value and the increase in %metmyoglobin over a 7 day display period was higher for the cold-boned IBF compared to the other groups. However, there was no significant difference in antioxidant enzyme activities, lipid and protein oxidation at day 10 of display. From the

results it can be concluded that the fast pH fall, slow temperature decline and the severe heat shortening in the IBF muscle from double-muscled Belgian Blue bulls under normal cooling practices, resulted in a pale colour and two-toning in the BF. Hot boning of the BF muscle can be seen as an appropriate solution for faster chilling and tackling problems with colour stability. However, hot boning of beef carcass had no significant effect on the CAT, SOD, and GSH-Px, as well as lipid and protein oxidation in meat.

The outcomes of this thesis suggest that GSH-Px and CAT have an important role in retarding oxidation, while SOD is probably not involved in protecting fresh meat against oxidation during storage. Moreover, the activity of antioxidant enzymes were influenced by the rate of pH and temperature fall from different muscles, time of display, as well as, the rate of chilling, with concomitant effects on meat oxidative stability.

De oxidatie van vetten, pigmenten en eiwitten speelt een belangrijke rol bij de sensorische en nutritionele kwaliteit van vlees en vleesproducten. Door de oxidatieprocessen te vertragen kan de houdbaarheid van deze producten verbeterd worden. Verdedigingsmechanismen tegen oxidatieprocessen *in vivo* omvatten onder andere de enzymen superoxide dismutase (SOD), catalase (CAT) en glutathion peroxidase (GSH-Px). De impact van deze antioxidant enzymen op het vertragen van oxidatieprocessen in spierweefsel post-mortem is echter niet helemaal duidelijk. De voornaamste doelstelling van dit doctoraal proefschrift was daarom het onderzoeken van de rol van antioxidant enzymen (SOD, CAT en GSH-Px) in het beschermen van vlees tegen oxidatieprocessen. Hiervoor werden specifieke remmers gebruikt tegen de enzymactiviteit. Ook werd het effect van temperatuur- en pH-daling post-mortem en spiertype onderzocht op de activiteit van deze enzymen in relatie met de oxidatieve stabiliteit van vlees en de vleeskwaliteit.

Hoofdstuk 1 bestaat uit een literatuurstudie over oxidatieve stress en antioxidant verdedigingsmechanismen *in vivo*, met nadruk op de antioxidant enzymen. Daarnaast werd een beknopt overzicht gegeven over vleeskwaliteit met nadruk op oxidatieprocessen in vlees.

In de experimenten beschreven in **hoofdstuk 2** werd de rol van GSH-Px, CAT en SOD in relatie tot oxidatieprocessen in rundvlees onderzocht en dit door hun activiteit post-mortem stil te leggen. Mercaptosuccinaat, natrium azide (NaN_3) en diethyldithiocarbamaat (DDC) werden gebruikt als specifieke remmers voor GSH-Px,

CAT en SOD respectievelijk, in drie verschillende dosissen versus een controle. De resultaten toonden aan dat de GSH-Px activiteit verlaagde, terwijl de TBARS waarden (vetoxidatie) en de eiwit carbonylcomponenten (eiwitoxidatie) stegen, naarmate hogere concentraties mercaptosuccinaat (tot 0.4 mM) werden toegediend. De CAT activiteit werd volledig geremd en de TBARS waarden stegen bij concentraties van 0.03 en 0.06% NaN_3 vergeleken met lagere concentraties, maar eiwitoxidatie werd niet beïnvloed. De SOD activiteit, TBARS waarden en eiwit carbonylcomponenten werden gereduceerd bij de hoogste concentratie DDC (1 mM). Hieruit werd besloten dat GSH-Px en CAT mogelijk een belangrijke rol hebben in het vertragen van vetoxidatie in vlees.

In **hoofdstuk 3** werd het effect van spiertype, het binnenste (inner) en buitenste (outer) deel van *M. Biceps femoris* (respectievelijk IBF en OBF) en *M. Longissimus dorsi* (LD) en de snelheid in pH- en temperatuurdaling op de activiteit van SOD, CAT en GSH-Px onderzocht tijdens blootstelling aan licht en lucht. De kleurstabiliteit, vetoxidatie en metmyoglobine vorming werden onderzocht in runderen van het Belgisch witblauw ras (n=40). De resultaten toonden aan dat de activiteit van CAT en GSH-Px lager en de vetoxidatie en metmyoglobine vorming hoger was in IBF vergeleken met OBF en LD, wat werd geassocieerd met een hogere eiwitdenaturatie en hittecontractie. De lagere activiteit van CAT en GSH-Px in IBF kan een gevolg zijn van meer denaturatie die werd geïnduceerd tijdens de langere hittecontractie periode. Echter, hittecontractie had geen invloed op de activiteit van SOD en het α -tocoferolgehalte van de spieren. De relaties die in deze studie werden gevonden tussen de enzymactiviteiten en vleeskwiteit suggereren dat de endogene antioxidant enzymen betrokken zijn in de bescherming van oxidatieve schade in vlees tijdens gekoelde bewaring.

In **hoofdstuk 4** werd de invloed van hoge post-mortem temperaturen op de activiteit van de enzymen en vleeskwiteit in *M. Longissimus dorsi* (LD) van varkens onderzocht. Er werden van 15 varkens LD stalen genomen van de linker en rechter karkashelft 30 min post-mortem. De LD stalen van de ene karkashelft werden 4 uur bij 40 °C in een heteluchtoven gehouden en vervolgens naar een koeler van 4 °C gebracht tot 24 post-mortem (vertraagde koeling), terwijl LD stalen van de andere karkashelft onmiddellijk in de koeler geplaatst werden (normale koeling). De vertraagde koeling resulteerde in PSE eigenschappen, hittecontractie, lagere eiwitoplosbaarheid en een lagere enzymactiviteit van CAT, GSH-Px en SOD vergeleken met de normale koeling 24 uur na snijden. De waarden van de kleurparameter L^* lagen hoger gedurende de 8 dagen blootstelling en de a^* waarden waren significant lager vanaf de vierde dag blootstelling bij de vertraagde koeling vergeleken met de normale koeling. De TBARS waarden waren lager na 8 dagen blootstelling bij de vertraagde koeling, maar er werden geen verschillen voor de eiwit carbonylcomponenten gevonden. Er werd besloten dat vertraagde koeling van varken LD een negatief effect heeft op de activiteit van de antioxidant enzymen en dat dit samen gaat met een inferieure kleurstabiliteit, maar dat vet- en eiwitoxidatie niet negatief beïnvloed werden.

In **hoofdstuk 5** werd het effect van warm versus koud ontbenen op de enzymactiviteit, kleurstabiliteit en vet- en eiwitoxidatie in IBF en OBF spieren van zeven runderen van het Belgisch witblauw ras onderzocht. De L^* waarde was hoger in de koud ontbeende IBF stalen vergeleken met de OBF stalen, wat resulteerde in tweetonigheid, terwijl de kleur niet verschilde in de IBF en OBF van de warm ontbeende stalen. Tegelijkertijd was de eiwitoplosbaarheid lager en de daling in a^* waarde en stijging in % metmyoglobine

hoger gedurende zeven dagen blootstelling voor de koud ontbeende IBF stalen vergeleken met de andere groepen. Er werden echter geen significante verschillen gevonden tussen de verschillende groepen in de enzymactiviteit van CAT, GSH-Px en SOD of voor vetoxidatie en eiwitoxidatie na 10 dagen blootstelling. Uit deze resultaten werd besloten dat de snelle pH daling, trage temperatuursdaling en de ernstige hittecontractie in de IBF van runderen van het Belgisch witblauw ras, gekoeld onder gangbare condities, resulteerde in een bleke kleur en tweetonigheid in de BF. Warm ontbenen van de BF spier kan gezien worden als een geschikte oplossing om snel te koelen en zo problemen met kleurstabiliteit aan te pakken. Echter, warm ontbenen had geen invloed op de enzymactiviteit van CAT, GSH-Px en SOD, noch op eiwit- en vetoxidatie in vlees.

De bevindingen van dit proefschrift suggereren dat GSH-Px en CAT een belangrijke rol spelen in het vertragen van oxidatieprocessen, terwijl SOD waarschijnlijk niet betrokken is bij het beschermen van vers vlees tegen oxidatie tijdens bewaring. Bovendien werd de activiteit van de antioxidant enzymen beïnvloed door de snelheid van pH- en temperatuursdaling in de verschillende spieren, snelheid van koelen en de duur van bewaring, met bijhorende effecten op de oxidatieve stabiliteit van het vlees.

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CURRICULUM VITAE

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PUBLICATIONS

A1 publications

Pastsart, U., De Boever, M., Claeys, E., & De Smet, S. (2013). Effect of muscle and post-mortem rate of pH and temperature fall on antioxidant enzyme activities in beef. Meat Science, 93, 681-686.

Pastsart, U., Claeys, E., & De Smet, S. Inhibition of antioxidant enzymes and the effect on lipid and protein oxidation in beef. *Animal Science Papers and Reports*. Submitted.

In preparation

Pastsart, U., Lescouhier, S., Claeys, E., & De Smet, S. Effect of hot boning on colour stability, antioxidant enzyme activities and oxidation in beef inner and outer *M. Biceps femoris*.

Pastsart, U., Lescouhier, S., Claeys, E., & De Smet, S. Effect of high post-mortem temperature on antioxidant enzyme activities and meat quality in pork loin.

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CONFERENCES, WORKSHOPS, SEMINARS

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56th International Congress of Meat Science and Technology (ICOMST), 15-20 August 2010, Jeju, South Korea. *Poster presentation.*

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